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GENETICS OF *STREPTOMYCES* AND OTHER ANTIBIOTIC-PRODUCING MICROORGANISMS

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GENETICS OF *STREPTOMYCES* AND OTHER ANTIBIOTIC-PRODUCING MICROORGANISMS*

Conference Chairman and Consulting Editor

WACLAW SZYBALSKI

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* This series of papers is the result of a conference on *Genetics of Streptomyces and Other Antibiotic-Producing Microorganisms* held and supported by The New York Academy of Sciences on January 6, 1959.

PREFACE

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The genetic analysis of *Streptomyces*, specifically the application of heterokaryotic and sexual-recombination analysis to these organisms, is a very young field, hardly more than three years old, but surprisingly international in character. It is gratifying, therefore, to see that almost every laboratory active in this area is represented in this monograph.

Two diverse stimuli presumably have attracted biologists to the genetic study of these microorganisms: (1) the streptomycetes are unique among the bacteria in their coenocytic structure, thus forming a morphogenetic link between the bacteria and the filamentous fungi; and (2) the importance of the streptomycetes as antibiotic producers makes economically imperative the development of methods for breeding and thereby improving the production strains. An apparent dichotomy of theoretical and practical interests is reflected in the literature. It is the object of this monograph, however, to demonstrate the complementary relationship of the different approaches.

While the volume is devoted primarily to *Streptomyces*, the work on penicillin production by filamentous fungi is included, since it formed a model for later studies on *Streptomyces*. On the other hand, lysogeny is discussed in the hope that this system may be useful in the demonstration of phage-mediated transfer of genetic information (transduction), although this type of genetic interaction as yet has not been demonstrated unequivocally for *Streptomyces*.

It is hoped that this collection of original articles, interspersed with discussions and polemics, will stimulate further investigation in the field, clarify the differences in interpretation, and weld the theoretical and practical interests.

INTRODUCTORY REMARKS

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Our present understanding of the manner in which genes control biochemical processes is based on studies of the inheritance of nutritional deficiencies in *Neurospora crassa*. These experiments, undertaken nearly twenty years ago by E. L. Tatum, now with The Rockefeller Institute, and G. W. Beadle, now with the California Institute of Technology, Pasadena, Calif., formed the foundation for the rapidly expanding field of modern microbial genetics. This monograph includes a paper by Tatum, who recently received the 1958 Nobel Prize in Medicine.

The techniques which Tatum and Beadle used to study the biochemical genetics of *Neurospora* have been applied to many organisms. Working together with his student and Nobel Prize corecipient, Joshua Lederberg, Tatum isolated many biochemical mutants of *Escherichia coli*. This work led to the discovery of sexual reproduction in bacteria.

These original studies were extended by scientists, working all over the world, to encompass the genetics of many traits in a variety of microorganisms. This monograph was prepared in order to record reports of outstanding workers in the very important field of the genetics of antibiotic-producing microorganisms. The monograph is, therefore, truly international in its authorship, and includes a contribution from S. I. Alikhanian, a distinguished scientist from the Union of Soviet Socialist Republics.

Part I. Genetics of *Streptomyces*

INTRODUCTION

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This monograph on the genetics of *Streptomyces* and other antibiotic-producing organisms is timely and of interest. Perhaps the major aim of this publication is to determine whether it is possible at present to employ a rational breeding program in these organisms, as has been done in higher organisms. The problem that is proposed here, however, differs sharply from the problems that plant and animal breeders have faced in the past. The *Streptomyces* and, in fact, all of the antibiotic-producing organisms are unique in involving organisms in which a meiotic cycle has not been observed. Thus, Protista breeders are faced with the intriguing problem: is it possible in the absence of the classic techniques of genetics to devise methods that would permit a rational breeding program that might be expected to yield high-producing strains?

The science of genetics in the last fifteen years has witnessed a dramatic change and, during this period, our understanding of the characteristics of genetic material has been enhanced greatly. Investigations of the genetics of diverse bacteria and viruses have underscored the fact that meiosis is not a prerequisite for genetic recombination, and the study of fungi has underscored the fact that reciprocal crossing-over is not a prerequisite to chromosomal recombination. Investigations of microorganisms of all sorts have enlarged our field of vision, have focused attention on still additional traits of genetic material, and have emphasized the fact that genetic recombination occurs in all forms of life. The problem that Protista breeders face, therefore, is not whether genetic recombination occurs, or does not occur; it does occur, and it reflects a basic and ubiquitous characteristic of genetic material in all forms of life. The problem that Protista breeders face is rather: can one use nonmeiotic recombination to give strains of microorganisms of superior traits?

In this monograph, the problems of the *Streptomyces* are considered first. The genetics of the *Streptomyces* is still a young field, and its problems are far from solved. However, it really is not necessary to question whether genetic interaction occurs in the *Streptomyces*. It is a foregone conclusion that it will occur, and it has been shown to occur. I am also sure that in the *Streptomyces*, as in the case of other bacteria, recombination will be found to occur in the absence of meiosis. The *Streptomyces* are evolutionarily related to that large group of organisms called true bacteria, and the hallmark of bacteria, and perhaps their most distinctive hallmark, is the fact that they represent a group of organisms that do not appear to have evolved a meiotic apparatus. However, it is known from the brilliant work of many investigators that recombination of genetic material in bacteria can occur in a number of different ways. It can occur by a process of cellular fusion, by a virus vector, or by a process of transformation. Thus, genetic interaction in *Streptomyces* occurs. The mech-

anisms that are involved or the factors required to permit recombination of genetic material from diverse strains of the *Streptomyces* still need to be understood. Does it occur by fusion of different strains and thus appear like the recombination observed in *Escherichia coli*? Can gene recombination be observed as a consequence of transduction, since it is known that the *Streptomyces* carry latent viruses? Also, there is the possibility of the direct uptake of DNA in the external medium, thus achieving recombination by a process similar to transformation. At the present time the answers to these problems are still under dispute, and these are the problems that will be examined and discussed here.

After the problem of the mechanism of genetic interaction is answered however, the possibilities of employing a nonmiotic type of recombination in the breeding of high producing strains remain to be considered. This problem is present in the *Streptomyces* but, as described elsewhere in these pages, it is also present in the imperfect fungi. In the *Streptomyces* there is a bacterial type of genetic interaction and, in the imperfect fungi, parasexuality. Basically, I believe, these phenomena are related closely, as they both involve a nonreciprocal type of recombination. The extent of recombination that can be achieved by these methods is limited compared to that which can be achieved in organisms having miosis. Under conditions of true sexuality, there is not only the opportunity for segmental and reciprocal recombination, but there is the possibility of the random assortment of chromosomes at the time of the reduction division. The methods considered here are very different, indeed. Will they yield equivalent results? At the present time it is not possible to determine whether they will, but it can be said with great assurance that our knowledge of the genetics of the *Streptomyces* and the other antibiotic-producing substances will, in the long run, prove of invaluable aid in the industrial exploitation of these organisms. Regardless of whether one can employ truly rational methods at the present time in the realm of Protista breeding, there can be no doubt that intensive genetic studies of these and related organisms will prove of benefit to science and to industry in the years ahead.

GENETIC INTERACTIONS WITHIN HETEROKARYONS OF STREPTOMYCETES*

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A heterokaryon is usually defined as a multinucleate cell carrying genetically or structurally different nuclei.[1] Because the cell is not a sharply delimited entity in microorganisms, heterokaryosis will be considered instead as genetic cooperation of diverse nuclei in a cytoplasmic field.[2] According to this latter definition, genetically different nuclei that are unable to interact through the cytoplasm will not be considered heterokaryotic even though there are no intervening cell walls.

Heterokaryons may be formed in several ways; a heterokaryon, for example, may arise as a consequence of a mutation in one or more of several nuclei in a cytoplasmic field. Alternatively, nuclear diversity can result from genetic reorganization by such processes as (1) autogamy, mitotic recombination, or haploidization within heterozygous diploids, (2) aneuploidy arising from nondisjunction, duplication, or deletion of some chromosomes, and (3) polyploidy. Heterokaryons, however, are usually thought of as recombinants containing genetically different nuclei brought together by cell fusion.

In *Neurospora crassa*, hyphal fusion does not lead necessarily to the formation of a functional, balanced, persistent heterokaryon.[3] The formation of a balanced heterokaryon is prevented because the cells of *N. crassa* in the vicinity of the site of hyphal fusion disintegrate following protoplasmic exchange. Moreover, dissociation occurs so rapidly under certain conditions that a balanced heterokaryon can not be demonstrated even though there is extensive hyphal fusion.[4] Incompatibilities, as determined by the inability of complementary auxotrophs to form functional heterokaryons, are also known in *Streptomyces coelicolor*.[5]

Compatibility

In our laboratory the terminology devised to describe sex compatibility in *Escherichia coli* has been adopted whenever applicable; two stocks, accordingly, have been considered crossable if selection of recombinant phenotypes was technically possible.[6] Fertility, therefore, has been used to denote the experimental production of recombinants by crossable parents, and compatibility has been reserved for the status of a stock with respect to the system herein described.

Complemental, auxotrophic strains of *S. coelicolor*, of common ancestry, yielded, in most cases, prototrophs when grown together;[7] certain mixtures involving strains of different origin, however, have not yielded recombinant phenotypes. Strain S207, for example, was crossable with strains derived from the same wild-type strain, but was not crossable with strains of different origin (TABLE 1). The infertility of S207 in combination with strains of di-

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verse origin was not the result of a general interstrain incompatibility because the closely related strain S206 was crossable with all strains of *S. coelicolor* with which it has been tested. Moreover, the dissimilarity of the growth-factor requirements made it unlikely that allelism was responsible for the inability to form prototrophs (TABLE 2). The fact that strains not crossable with

TABLE 1

RELATIVE NUMBER OF PROTOTROPHS FORMED BY AUXOTROPHIC PAIRS OF *STREPTOMYCES COELICOLOR*

Strain No.	23	14	S207	S206	S125	S204	S203	S202	S205	Compatibility status*
S202	++++	++	−	+	++++	++	++	−	+++	C
S203	++++	+	−	+	++++	++	−	++	++	C
S204	X	X	−	+	+++	−	++	++	++	C
S205	X	X	−	+	++	++	++	+++	−	C
S206	X	X	+++	−	+++	+	+	+	+	B
S207	X	X	−	+++	+	−	−	−	−	A
S125	X	X	+	+++	−	+++	++++	++++	++	B
14	+++	−	X	X	X	X	+	++	X	C
23	−	+++	X	X	X	X	++++	++++	X	C

Key: X, not tested; −, 0 prototrophs formed; +, 5 to 25 prototrophs/plate; ++, 10 to 50 prototrophs/plate; +++, 30 to 200 prototrophs/plate; ++++, over 200 prototrophs/plate.

* The auxotrophic pairs were grown together on complete medium for 6 days and then replica-plated to minimal medium. Minimal plates were scored after 5 days.

TABLE 2

PRINCIPAL STOCKS: THEIR PHENOTYPES AND ORIGIN

Strain No.	Origin	Phenotype*
S199	nature	prototrophic V^s
S202	S199	cys-pro-V^s
S203	S199	ura-arg-V^s
S204	S199	met-V^s
S16	NRRL-B-1257, Peoria, Ill.	prototrophic V^r
S206	S16	arg-V^r
S207	S16	OHpro-V^r
S125	S16	met-V^r
14	G. Sermonti, Rome, Italy	pro-glu-V^s
23	G. Sermonti, Rome, Italy	met-his-V^s
S205	14	his-V^s

* V^r/V^s: resistance/sensitivity to actinophage MSP-7.

Cys-, pro-, ura-, met-, arg-, OHpro-, glu-, his-, require added cystine, proline, uracil, methionine, arginine, hydroxyproline, glutamate, or histidine, respectively.

strain S207 were crossable with one another provided additional evidence that the observed infertility was not the result of allelism.

With the exception of strain S207, the primary feature of this compatibility system was self-fertility. The observed compatibility may be symbolized thus:

A + B → fertile
B + C → fertile
A + C → sterile
A + A → not tested
B + B → fertile
C + C → fertile

for which strains S207, S206, and S203 have been adopted as the standard testers for A, B, and C, respectively.

Mechanism of Incompatibility

Reconstruction experiments were performed in order to determine if incompatibility resulted from adverse selection of recombinants. In these experiments, known numbers of wild-type spores were dispensed to complete medium, along with spores of each member of an incompatible pair. These three-membered cultures were grown together for six days before they were transferred to minimal medium by replica plating. The wild type was recovered efficiently, thereby confirming the fact that, should prototrophs be

TABLE 3
EFFICIENCY OF DETECTING PROTOTROPHIC GROWTH IN MIXED CULTURES OF INCOMPATIBLE PAIRS

Mixture*	Size of mixed culture inoculum	No. prototrophic colonies recovered by replica-plating
S199 alone	83	91
S207 alone	5×10^5	0
S205 alone	4×10^5	0
S205 + S207	9×10^3	0
S205 + S207	9×10^4	0
S205 + S207	9×10^5	0
S205 + S207 + S199	9×10^3 83	86
S205 + S207 + S199	9×10^4 83	79
S205 + S207 + S199	9×10^5 83	84
S205 + S207 + S199	9×10^4 218	236

* Mixed cultures were grown on complete medium for 6 days and then replica-plated to minimal medium. Minimal plates were scored after 5 days.

formed by nuclear or genic recombination, the testing method would have detected them (TABLE 3).

An unusual feature of the recombinational systems in *E. coli* and *Pseudomonas aeruginosa* was the infectious nature of a fertility factor.[8] In these cases, self-sterile F− strains were converted to self-fertile F+ strains by culturing the two types together. Experiments, therefore, were conducted to determine if the compatibility status of strain S207 was changed during growth in a fertile combination. Accordingly, strains S207 and S206 were grown together on complete medium for 6 days, after which the parental S207 type was recovered. After the growth-factor requirement was substantiated, strain S207 was grown in combination with S205; as was found previously, less than 1 prototroph was formed per 10^8 viable units. Strain S207, therefore, did not become infected with a compatibility factor during growth in mixture with S206.

Incompatibility could have resulted from failure of the hyphae to fuse, or persistent genetic interaction could have been prevented after hyphal fusion,

FIGURE 1. Hyphal fusion between a compatible pair of *Streptomyces coelicolor*. Dark phase-contrast microscopy.

FIGURE 2. Hyphal fusion between an incompatible pair of *Streptomyces coelicolor* mutants. Dark phase-contrast microscopy.

as was the case for *N. crassa.* To ascertain whether hyphal fusion occurred between incompatible pairs, the strains were sown on agar as parallel rows 1 mm. apart. After 5 to 7 days' incubation the plates were examined microscopically under low-power magnification; areas that seemed to show hyphal fusion between two filaments, each of which definitely originated from a different parental streak, were cut from the agar. The small agar blocks were then placed on a microscope slide with a drop of water, covered with a cover slide, and melted. These preparations were examined under dark-phase contrast microscopy at a magnification of 2000 diameters. Hyphal fusion occurred extensively between compatible pairs and was common between incompatible strains as well (FIGURES 1 and 2).

Heterokaryosis in Streptomyces griseus

Pairs of complemental, auxotrophic variants of *S. griseus* that were grown together on nutritionally limiting medium produced prototrophic tufts. The genetic composition of these colonies was determined by the following procedure: the prototrophic mycelia were suspended in complete broth and then minced; the resulting hyphal suspension was distributed as small drops; single hyphal segments were isolated by micromanipulation; 97 of the single hyphal fragments formed microcolonies in complete broth; these small mycelia were transferred to complete agar in order that aerial hyphae would develop; the spores from each of 61 colonies yielded both parental types; spores from each of the remaining 36 colonies were of one or the other parental phenotype.[9] It thereby was proved that a single hyphal segment could contain both of the unaltered parental genomes; hence the prototrophic tufts were considered heterokaryotic. In an experiment in which the hyphal fragments were isolated in minimal broth instead of complete broth and the resulting microcolonies were transferred to minimal agar rather than complete agar, 13 of 15 isolates formed growth-factor independent colonies. If the hyphal fragments were isolated in complete broth instead and the resulting minute mycelia transferred to minimal agar, only 6 of 29 microcolonies were able to produce normal-sized colonies within 4 days. On the fifth day, yeast extract was added to the nongrowing microcolonies so that normal-sized colonies were produced; of the 23 isolates unable to grow on minimal medium, both parental types were recovered among spores from each of 15 colonies. The remaining 8 colonies each yielded spores of one or the other parental phenotype. Thus the prototrophic character was transmitted frequently through hyphal segments ranging from 3 to 15 μ, but the medium in which the hyphae were grown after isolation influenced the expressivity of the genetic markers.

In contrast to the vegetative hyphae, spore preparations rarely perpetuated the recombinant phenotype; less than 0.1 per cent of the spores from a prototrophic colony developed on minimal medium. Microscopic examination of the spore preparations revealed that the incidence of prototrophy corresponded closely to the frequency of mycelial fragments and intact chains of spores. Even though growth-factor independence was rarely, if ever, transmitted through the spores, certain infrequent nonparental auxotrophs were perpetuated regularly by spores. Those colonies whose nutritional requirements were not

determined definitively were designated anomalous heterokaryons. One class of anomalous heterokaryons, which resulted from crosses involving doubly auxotrophic parents, would grow on minimal medium supplemented with any 3 of the 4 growth factors required by the parents. Both of the parental types were recovered eventually as colonies or sectors during serial subculture of the anomalous heterokaryons; therefore their spores contained both parental genomes. As yet, no definitive interpretation concerning the nature of these anomalous heterokaryons has been presented.

Heterokaryons that regularly produced homokaryotic spores, as was the case with *S. griseus*, made it possible to determine if all the spores in a single chain were derived from a single nucleus or, rather, if the spores in an individual chain were of diverse origin. Two types of experiments were undertaken to resolve this point: on the one hand, intact chains of spores were isolated by micromanipulation and the colonies that developed from single spore chains were analyzed; alternatively, intact chains were first isolated and then individual spores were picked from the chain. The latter approach was unsuccessful for the most part because only about 10 per cent of the isolated single spores were viable. The low viability of the individual spores probably resulted from the extensive mechanical agitation required to free and isolate the single spores. The vast majority of the spore chains were of one or the other type; approximately 19 per cent of the chains, however, were mixed (TABLE 4). Contamination probably was not responsible for the significant level of mixed spore chains. It was equally unlikely that all the chains were mixed, but differential survival produced an abundance of one-type chains.

TABLE 4

PHENOTYPE OF SPORE CHAINS DERIVED FROM HETEROKARYOTIC COLONIES OF *S. GRISEUS*

No. chains* isolated	No. single spores viable	try-pu$_2$-	arg-pu$_1$-	Mixed
26	not determined	12	9	5
7	3	3	0	no
	3	3	0	no
	3	3	0	no
	3	0	3	no
	4	4	0	no
	4	3	1	yes
	6	0	6	no

* Prototrophs were obtained on minimal medium after 12 days incubation; the inoculum consisted of 10^6 spores/plate from both the arg-pu$_1$-parent and the try-pu$_2$-parent.

Heterokaryosis in Streptomyces coelicolor

Complemental, auxotrophic pairs of *Streptomyces coelicolor* rarely produced prototrophic outgrowths when plated together on minimal medium. If two biochemical mutants were grown together on complete medium before being transplanted to minimal medium, prototrophs were obtained regularly. Spores from prototrophic recombinants of *S. coelicolor* grew readily on minimal medium,[7,10] whereas spores from prototrophic recombinants of *S. griseus* yielded

regularly one or the other parental type. Although the inheritance of growth-factor requirements has been followed throughout several subcultures, heretofore unselected markers have not been studied adequately.[11] In some of the crosses reported here, susceptibility to bacteriophage and streptomycin has been used as unselected characters.

Susceptibility to bacteriophage was determined as follows: a loopful of a bacteriophage suspension of known titer was streaked on complete medium; the streptomycete to be tested was then streaked across the phage. If the bacterium grew uninterrupted through the phage streak, the organism was considered resistant; if the streptomycete was lysed at the phage-host intersection, the organism was scored as sensitive.

Three crosses were analyzed in which the parents differed with respect to phage susceptibility; these crosses were between S206 + S205, S206 + S204, and S207 + S206*. Strain S206* was a mutant of S206 selected for resistance

TABLE 5

SEGREGATION OF BACTERIOPHAGE SUSCEPTIBILITY AMONG PROTOTROPHS OF *S. COELICOLOR*

Combinations	Phenotypes	No. tested	V^s[1]	V^r
S206 alone	OHpro-V^r	81	0	81
S206* alone	OHpro-V^rP^r	76	0	76
		76(P)[2]	0(P)	76(P)
S207 alone	arg-V^rP^s	72	0	72
		72(P)	72(P)	0(P)
S204 alone	met-V^s	85	85	0
S205 alone	his-V^s	60	60	0
S205 + S206	see above	235	235	0
S204 + S206	see above	115	115	0
S206* + S207	see above	92(P)	92(P)	0(P)

[1] V^s/V^r denotes sensitive/resistance to bacteriophage MSP-7.
[2] P denotes status with respect to bacteripohage MSP-11 rather than MSP-7.

to bacteriophage MSP-11. The selected prototrophs were purified on complete medium by two serial subcultures from single colonies before phage susceptibility was determined. All of the combinations yielded only phage-sensitive prototrophs (TABLE 5).

The behavior of streptomycin susceptibility in crosses between S203 and Sermonti's strain 23 has been followed during several subcultures. It was noteworthy that the expressivity of certain characters, that is, growth-factor requirements and streptomycin susceptibility, varied according to the nutritional markers selected. Mixed cultures of strain 23 (met-his-S^R) and S203 (ura-arg-S^s), for example, were replica-plated to minimal medium; the recombinants so obtained were isolated and purified prior to testing for susceptibility to streptomycin. All of the prototrophs were found to be sensitive. Contrariwise, when the selective medium was minimal agar supplemented with arginine and methionine, all of the purified recombinant colonies were found to be initially resistant to streptomycin. The expression of the streptomycin character, moreover, was not inherited regularly during serial subculture of spores from a single colony (TABLES 6, 7, and 8). Recombinants variably and infrequently yielded parental dissociates; the high incidence of dissociation

TABLE 6

SEGREGATION OF *STREPTOMYCES COELICOLOR* RECOMBINANTS OBTAINED BY MIXED GROWTH OF COMPLEMENTARY AUXOTROPHS

auMHS^s × AUmhS^{R*}

↓

Grow Together on Complete Medium

↓

Replica Plate to HA†

↓

Spores from Individual Colonies Are Plated onto Differential Media

Subculture‡		No. strains	Derived from	Differential media§							Phenotype
				minimal	complete	SM	MHA	MHU	AUM	AUH	
Original		30	replica plates	−	+	−	+	+	+	+	anomalous S^s
First	a	28	original	+	+	+	+	+	+	+	AUMHS^r
	b	2	original	−	+	−	+	+	+	+	anomalous S^s
Second	a	28	1a	+	+	+	+	+	+	+	AUMHS^r
	b	1	1b	−	+	−	−	−	+	+	auMHS^s‖
	c	1	1b	−	+	+	+	+	−	−	AUmhS^r
Third	a	10	2a	−	+	−	−	−	+	+	auMHS^s
	b	7	2a	−	+	+	+	+	−	−	AUmhS^r
	c	3	2a	−	+	+	+	+	+	+	anomalous S^R
	d	3	2a	−	+	−	+	+	+	+	anomalous S^s
	e	1	2a	+	+	+	+	+	+	+	AUMHS^r
	f	4	2a	+	+	−	+	+	+	+	AUMHS^s
Fourth	a	9	3c	−	+	−	−	−	+	+	auMHS^s
	b	4	3d	−	+	+	+	+	−	−	AUmhS^r
	c	5	3d	−	+	−	−	−	+	+	auMHS^s
	d	3	3e	−	+	+	+	+	−	−	AUmhS^r
	e	1	3f	+	+	−	+	+	+	+	AUMHS^s
	f	1	3f	−	+	+	−	−	+	+	auMHS^r
	g	1	3f	−	+	−	+	+	−	−	AUmhS^s
	h	6	3f	−	+	−	+	+	−	−	auMHS^s
	i	3	3f	−	+	+	+	+	−	−	AUmhS^r
Fifth	a	2	4e	−	+	−	−	−	+	+	auMHS^s
	b	1	4e	+	+	−	+	+	+	+	AUMHS^s blue
	c	1	4e	+	+	−	+	+	+	+	AUMHS^s pink
	d	1	4f	−	+	−	−	−	+	+	auMHS^s
Sixth	a	4	5b	−	+	+	+	+	−	−	AUmhS^r
	b	1	5b	−	+	−	−	−	+	+	auMHS^s
	c	1	5b	−	+	−	−	−	−	−	aumhS^s
	d	4	5b	+	+	−	+	+	+	+	AUMHS^s blue
	e	1	5c	+	+	−	+	+	+	+	AUMHS^s pink
Seventh	a	3	6c	−	+	−	−	−	+	+	auMHS^s
	b	4	6d	+	+	−	+	+	+	+	AUMHS^s blue
	c	1	6e	+	+	−	+	+	+	+	AUMHS^s pink
Eighth	a	1	7c	−	+	−	+	+	−	+	AUMhS^s pink
	b	1	7c	+	+	−	+	+	+	+	AUMHS^s pink
	c	4	7b	+	+	−	+	+	+	+	AUMHS^s blue
Ninth	a	4	8c	+	+	−	+	+	+	+	AUMHS^s blue
	b	1	8b	+	+	−	+	+	+	+	AUMHS^s pink
	c	1	8a	−	+	−	+	+	−	+	AUMhS^s pink
Tenth	a	4	9a	+	+	−	+	+	+	+	AUMHS^s blue
	b	2	9b	−	+	−	+	+	+	+	anomalous S^s pink
	c	2	9c	−	+	−	+	+	+	+	anomalous S^s pink

* A/a, U/u, M/m, or H/h: independent/dependent for exogenous arginine, uracil, methionine, or histidine, respectively.

† MHU, MHA, AUM, HA, or AUH: minimal medium supplemented with methionine-histidine-uracil, methionine-histidine-arginine, arginine-uracil-methionine, histidine-arginine, or arginine-uracil-histidine, respectively.

‡ Subculture inoculum consisted of spores from a single colony that was grown on complete medium.

§ Symbols: +, growth equivalent to complete medium; ±, growth 5 to 20 per cent of complete control; −, no growth.

‖ Parental types were confirmed by one further subculture.

TABLE 7

SEGREGATION OF *STREPTOMYCES COELICOLOR* PROTOTROPHS OBTAINED BY MIXED GROWTH OF COMPLEMENTARY AUXOTROPHS

auMHS^s × AUmhS^r*

↓

Grow Together on Complete Medium

↓

Replica Plate to Minimal Medium

↓

Spores from Individual Colonies Are Plated onto Differential Media

Subculture†		No. strains	Derived from	Differential media‡§							Phenotype
				minimal	complete	SM	MHA	MHU	AUM	AUH	
Original		55	replica plates	+	+	−	+	+	+	+	AUMHS^s
First	a	42	original	+	+	−	+	+	+	+	AUMHS^s
	b	6	original	−	+	+	+	+	−	−	AUmhS^r
	c	6	original	−	+	−	−	−	+	+	auMHS^s‖
	d	1	original	−	+	−	+	+	+	+	anomalous S^s
Second	a	42	1a	+	+	−	+	+	+	+	AUMHS^s
	b	1	1d	−	+	−	+	+	+	+	anomalous S^s
Third	a	36	2a	+	+	−	+	+	+	+	AUMHS^s
	b	6	2a	+	+	+	+	+	+	+	AUMHS^r
	c	1	2b	−	+	−	−	−	+	+	auMHS^s
Fourth	a	6	3b	−	+	+	+	+	−	−	AUmhS^r
	b	6	3a	−	+	−	−	−	+	+	auMHS^s
	c	30	3a	+	+	±	+	+	+	+	AUMHS^s AUMHS^r
Fifth	a	2	4c	+	+	+	+	+	+	+	AUMHS^r
	b	28	4c	+	+	−	+	+	+	+	AUMHS^s
Sixth	a	2	5a	−	+	−	+	+	−	−	AUmhS^s
	b	51	5b**	+	+	−	+	+	+	+	AUMHS^s
	c	4	5b**	−	+	−	−	−	+	+	auMHS^s
	d	1	5b**	−	+	−	+	+	+	+	anomalous S^s
Seventh	a	2	6a	−	+	+	+	+	−	−	AUmhS^r
	b	3	6d††	−	+	−	+	−	+	+	aUMHS^s
	c	9	6b	−	+	−	−	−	+	+	auMHS^s
	d	3	6b	+	+	+	+	+	+	+	AUMHS^r
	e	38	6b	+	+	−	+	+	+	+	AUMHS^s
	f	1	6b	−	+	−	−	−	−	−	aumhS^s
Eighth	a	3	7b	−	+	−	+	−	+	+	aUMHS^s
	b	3	7d	−	+	+	+	+	−	−	AUmhS^r
	c	38	7e	+	+	−	+	+	+	+	AUMHS^s
	d	3	7f§§	+	+	+	+	+	+	+	AUMHS^r
	e	1	7f§§	+	+	−	+	+	+	+	AUMHS^s
	f	5	7f§§	−	+	−	−	−	−	−	aumhS^s

* A/a, U/u, M/m, or H/h: independent/dependent for exogenous arginine, uracil, methionine, or histidine, respectively.

† Subculture inoculum consisted of spores from a single colony that was grown on complete medium.

‡ MHU, MHA, AUM, or AUH: minimal medium supplemented with methionine-histidine-uracil, methionine-histidine-arginine, arginine-uracil-methionine, or arginine-uracil-histidine, respectively.

§ Symbols: +, growth equivalent to complete medium; ±, growth 5 to 20 per cent of complete control; −, no growth.

‖ Parental types were confirmed by one further subculture.

** Two colonies were picked from each.

†† Three colonies were picked.

§§ Nine colonies were picked.

reported herein was due to the selection of atypical colonies after the third subculture.

A pink prototroph was obtained in our laboratory as a dissociate from a blue, nutritionally independent colony that was derived from a cross involving

TABLE 8

SEGREGATION OF *STREPTOMYCES COELICOLOR* RECOMBINANTS OBTAINED BY MIXED GROWTH OF COMPLEMENTARY AUXOTROPHS

auMHS^s × AUmhS^{r*}

Grow Together on Complete Medium

Replica Plate to AM†

Spores from Individual Colonies Are Plated onto Differential Media

Subculture‡	No. strains	Derived from	Differential Media§							Phenotype
			minimal	complete	SM	MHA	MHU	AUM	AUH	
Original	24	replica plates	±	+	+	+	+	+	+	AUMHS^r anomalous S^r
First	24	original	±	+	+	+	+	+	+	AUMHS^r anomalous S^r
Second a	2	1	−	+	−	−	−	+	+	auMHS^s‖
b	2	1	−	+	+	+	+	−	−	AUmhS^r
c	10	1	−	+	+	+	+	+	+	anomalous S^r
d	10	1	−	+	−	+	+	+	+	anomalous S^s
Third a	10	2c	−	+	+	+	+	+	+	anomalous S^r
b	10	2d	−	+	−	+	+	+	+	anomalous S^s
Fourth a	8	3a	−	+	+	+	+	−	−	AUmhS^r
b	2	3a	−	+	−	+	+	+	+	anomalous S^s
c	10	3b	−	+	−	+	+	+	+	anomalous S^s
Fifth a	2	4b	−	+	+	+	+	−	−	AUmhS^r
b	1	4c	−	+	+	+	+	−	−	AUmhS^r
c	9	4c	−	+	−	+	+	+	+	anomalous S^s
Sixth a	2	5c	−	+	−	−	−	+	+	auMHS^s
b	7	5c	−	+	−	+	+	+	+	anomalous S^s
Seventh a	7	6b	−	+	−	−	−	+	+	auMHS^s

* A/a, U/u, M/m, or H/h: independent/dependent for exogenous arginine, uracil, methionine, or histidine, respectively.

† AM, MHA, MHU, AUM, or AUH: minimal medium supplemented with arginine-methionine, methionine-histidine-arginine, methionine-histidine-uracil, arginine-uracil-methionine, or arginine-uracil-histidine.

‡ Subculture inoculum consisted of spores from a single colony that was grown on complete medium.

§ Symbols: +, growth equivalent to complete medium; ±, growth 5 to 20 per cent of complete control; −, no growth.

‖ Parental types were confirmed by one further subculture.

two blue parental types (TABLE 6); the origin of nonparental auxotrophs among segregants from recombinants had been noted by us previously.[10] Of 1416 auxotrophic dissociates isolated from a cross between an arg-glu-strain and a met-try-strain, both derived from wild-type S16, 49 auxotrophic strains bearing characteristics not recognized in either parent were found. Of these new markers, 18 were for cystine, 23 for adenine, 3 for lysine, 2 for proline, 2 for

uracil, and 1 for valine. The origin of these new mutants has not been determined adequately.

Heterokaryosis and Systematics

It has long been recognized that a streptomycete unable to form sporulating aerial hyphae cannot be distinguished from a nocardia.[12] More recently it was shown that several members of the genus *Nocardia* do indeed sporulate;[13, 14] therefore, the basis for separating the genera *Nocardia* and *Streptomyces* has become even more vague. It has been emphasized that the taxonomic difficulties encountered in this group are not limited to the generic level; the problem of speciation has also been particularly troublesome in the genus *Streptomyces*.[15] Of the many criteria suggested to facilitate species identification in the streptomycetes,[16] few reliable tests have been found. In the imperfect fungi, hyphal anastomosis occasionally has been used as an aid in speciation,[17] because several workers agreed that hyphal fusion occurred only between members of the same species.[18]

Hyphal anastomosis was not, in our laboratory, a reliable basis for speciation in the streptomycetes because balanced heterokaryons were obtained between *S. griseus* and *S. cyaneus* and between *S. griseus* and *S. venezuelae*. Although balanced heterokaryons have not been obtained between *S. griseus* and *S. olivaceus*, hyphal fusion has been observed; neither balanced heterokaryons nor hyphal fusion has been demonstrated in combinations of *S. griseus* with *S. coelicolor*. In the streptomycetes, biological affinities as determined by hyphal fusion and cross susceptibility to bacteriophages were similar; hyphal fusion, therefore, might be useful in generic determinations and in establishing group species. It must be noted that failure of hyphae to fuse is not proof of biological dissimilarity.

Discussion

From the data presented here and from similar work conducted by others, certain conclusions may be drawn concerning the genetic structure and processes of the streptomycetes.[10, 11] With *S. griseus* the mutation rates for recessive characters are identical to the mutation rates in accepted haploid microorganisms; it is difficult, however, to distinguish between haploidy and diploidy accompanied with frequent autogamy. Moreover, spores from *S. griseus* heterokaryons are of one or the other parental phenotype; the spores, therefore, are either uninucleate of homokaryotic. The results obtained with X-irradiation inactivation are also consistent with the hypothesis that the spores of *S. griseus* are uninucleate and haploid.[19]

The situation in *S. coelicolor* in many respects is quite different from the situation in *S. griseus*; at least some spores of *S. coelicolor*, for example, contain more than one genome. Although the prototrophic phenotype of recombinants is frequently perpetuated through the spores, other phenotypes do arise during subculture; the wild type and parental types, however, are the most frequent products of dissociation.

Difficulty is encountered in developing an acceptable hypothesis to account for all of the observed genetic behavior because (1) it is not possible to reproduce

data quantitatively and (2) some expressions are not inherited predictably. These complexities have resulted in a diversity of interpretations based on very similar data.

Summary

Most pairs of growth factor-dependent mutants of *S. griseus* or *S. coelicolor* interacted to form nutritionally independent recombinants. Certain combinations of *S. coelicolor* mutants of diverse origin, however, were not fertile; the observed incompatibility was not the result of a general interstrain barrier, nor were the growth-factor requirements allelic. Hyphal fusion occurred between hyphae of sterile pairs.

With *S. griseus*, the recombinant phenotype was transmitted regularly by hyphal segments, but the spores resembled one or the other parent. The majority of the conidiospore chains from recombinant colonies contained spores of a single type; however, a significant number of mixed spore chains were found.

Recombinants of *S. coelicolor*, unlike *S. griseus*, regularly produced prototrophic spores. During serial subculture of recombinant clones of *S. coelicolor* parental phenotypes were recovered. Moreover, in crosses involving parents of different bacteriophage susceptibility, only sensitive prototrophs were detected.

Hyphal anastomosis occurred between different species of the genus *Streptomyces*. Biological affinities, as determined by hyphal fusion and cross susceptibility to bacteriophages, were similar.

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Discussion of the Paper

K. F. Gregory (*Ontario Agricultural College, Guelph, Canada*): The ability of spores of *Streptomyces coelicolor* to perpetuate the anomalous auxotrophic heterokaryons described in this species appears to be somewhat incompatible with the conclusion that the auxotrophic nature of these strains results from a major imbalance in the proportion of parental nuclei. Since the proportion of parental nuclei in various anomalous auxotrophic heterokaryons has been reported to range from 1:10 to 1:1000, one might reasonably expect that the minimal plating unit capable of perpetuating the anomalous auxotrophic heterokaryon would contain from 10 to 1000 nuclei, surely improbable figures for streptomycete conidia.

S. G. Bradley: It is unlikely that a single spore contains 10 to 1000 nuclei; however, one class of nuclei may undergo mitosis before the other class. In a heterokaryotic binucleate spore, a lead of 3 nuclear divisions will produce a mycelium with a tenfold excess of one nuclear type; a lead of 6 to 7 generations will yield a hundredfold excess of one nuclear type; etc. Other variables affecting nuclear division can cause similar results.

HETEROKARYOTIC COMPATIBILITY, METABOLIC COOPERATION, AND GENIC RECOMBINATION IN *STREPTOMYCES**

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Despite their great economic importance, no studies on genetic interaction among streptomycetes were undertaken until recently, when a number of papers appeared almost simultaneously. A model was provided by the work on genetic recombination in bacteria, initiated by Lederberg and Tatum (1946). In spite of their moldlike appearance, the streptomycetes are related closely to the Gram-positive bacteria, especially in the biochemical composition of their cell walls. The properties of sensitivity to lysozym and actinophage, which also relate to the cell wall, together with their diminutive dimensions clearly distinguish the streptomycetes from true filamentous molds. It is perhaps paradoxical, therefore, that the cell envelope is also responsible for the moldlike morphology of these organisms, through failure of cross-wall production concomitant to nuclear division. The resulting coenocytic mycelium dictates many of the properties of the streptomycetes, including their genetic behavior. In this context, the genetic studies on filamentous fungi, including *Aspergillus* and *Penicillium* (Pontecorvo, 1953), undoubtedly provided another stimulus for similar work on *Streptomyces*.

It would be redundant to review here the earlier studies on genetic interactions among streptomycetes, since the work of most of the original contributors is included in this monograph. This paper, therefore, will be limited to a survey of our published and unpublished work.

Isolation of Mutants and Their Stability

For the development of marked strains of *Streptomyces*, the methods applied earlier to the genetic study of bacteria and of filamentous fungi were combined. The first step was the preparation of a homogeneous suspension of conidia. These were harvested from well-sporulated growth on agar by means of a glass spreader after flooding the surface with a saline solution containing 0.05 per cent Triton WR-1339.‡ The resulting heavy suspension was filtered through Whatman No. 2 filter paper in small Buchner funnels. The result was a uniform, well-dispersed suspension of 10^8 to 10^9 conidia/ml., practically free of mycelium and intact conidial chains or clumps.

As the second step in the procedure, the number of mutants in the suspension was increased under the action of an appropriate mutagen, usually ultraviolet light. The conidia were irradiated in water suspension to a survival level of 1 to 50 per cent.

To facilitate their isolation, the presumptive mutants in the conidial suspension were concentrated by the filtration technique. This step was based

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‡ Product of Rohm and Haas Co., Philadelphia, Pa.

on the assumption that, in a minimal medium containing glucose as the only carbon source, prototrophic conidia should germinate readily while spores of induced auxotrophic mutants should show, at best, a much longer lag period. The former, after producing some mycelial growth, should be largely retained by filter paper (Whatman No. 2), while the latter should pass through. For mutations showing a phenotypic lag, a single subculture on nutrient agar after irradiation, with the production of a secondary crop of conidia, would be required. The filtration technique was evaluated on an artifical mixture of prototrophic and auxotrophic conidia, with a resulting increase of between tenfold and one thousandfold in the proportion of auxotrophic components. The procedure was based on the method developed by Fries (1947) for the fungus *Ophiostoma.*

The velveteen replica-plating technique (Lederberg and Lederberg, 1952) or its modification, multi-pin replication (Szybalski, 1956), was employed for the isolation of auxotrophic mutants in the fourth step of the procedure. Most of the *Streptomyces* strains formed colonies suitable for the replication procedure. Multipin replication permitted testing of as many as and more than 1000 colonies/plate, while 50 to 100 colonies/plate was the upper limit for velveteen replication. Colonies replicating on nutrient agar, but failing to grow on minimal medium, were isolated, purified, and characterized as to growth requirements by the procedure summarized by Lederberg (1950). These were the concluding steps in the isolation of mutants with single nutritional markers. To obtain mutants with multiple deficiencies, the whole procedure was repeated using the singly marked strain and minimal media enriched by its growth factor.

During plating and replication, several nonnutritional mutants were isolated by visual observation. These were characterized by altered pigment production, loss of the ability to sporulate, and certain readily apparent morphologic changes.

For the isolation of mutants resistant to antimicrobial agents, the irradiated conidia were first subcultured on complete medium and subsequently on gradient plates (Szybalski, 1952) containing inhibitory concentrations of toxic agents. Colonies growing beyond the boundary of inhibition were subcultured on gradient plates with gradually increasing concentrations of the drug until it was possible to isolate organisms that would grow at drug levels at least tenfold higher than that required to inhibit completely the sensitive parent. *Streptomyces* proved to be quite sensitive to many antibacterial antibiotics, although there was considerable variation among species. Resistance markers for the following drugs were found to be most useful: bacitracin, carbomycin, catenulin, cinnamycin, kyostacin, mycomycetin, neomycin, novobiocin, streptomycin, tetracycline, tyrocidine, and viomycin, among others.

Table 1 lists the mutants employed in the present study.

Mutant stocks were either lyophilized or stored on nutrient agar slants at 2° C. The stability and viability of the refrigerated stocks were evaluated after 1 to 2 years' storage. Most of the cultures appeared to be viable with auxotrophic requirements unchanged. The mutants of *S. spheroides*, however, were no longer viable, and the *S. coelicolor* auxotrophs showed large accumulations of prototrophic revertants.

TABLE 1
LIST OF MUTANT STRAINS

Code	Derived from strain	Markers*
3E12	*S. albus* No. 618	met$^-$
14D1-1	*S. coelicolor* (R & M, G. Sermonti)	met$^-$, his$^-$, pigm$^-$
14D1-2	*S. coelicolor* (R & M, G. Sermonti)	met$^-$, his$^-$, pigm$^+$, SM^r
14D2-1	*S. coelicolor* (R & M, G. Sermonti)	pro$^-$, glu$^-$, pigm$^-$, SM^r
14D1	*S. coelicolor* (R & M, G. Sermonti)	met$^-$, his$^-$, pigm$^+$
14D2	*S. coelicolor* (R & M, G. Sermonti)	pro$^-$, glu$^-$, pigm$^-$
12D1	*S. coelicolor* (D. A. Hopwood)	his$^-$
12D2	*S. coelicolor* (D. A. Hopwood)	met$^-$
12D3	*S. coelicolor* (D. A. Hopwood)	his$^-$, pha$^-$, SM^r, pigm$^+$
12D4	*S. coelicolor* (D. A. Hopwood)	met$^-$, pha$^-$, SM^r, pigm$^+$
13D1	*S. coelicolor* NI 9021	his$^-$, pigm$^+$
13D1-1	*S. coelicolor* NI 9021	his$^-$, cys/met$^-$, pigm$^+$
13D2	*S. coelicolor* NI 9021	ser/gly$^-$
13D3	*S. coelicolor* NI 9021	met$^-$
13D4	*S. coelicolor* NI 9021	arg$^-$
13D5	*S. coelicolor* NI 9021	glu$^-$
13D6	*S. coelicolor* NI 9021	(isl + val)$^-$
13D7	*S. coelicolor* NI 9021	met$^-$
13D8	*S. coelicolor* NI 9021	isl$^-$
6F2	*S. fradiae* No. 3535	arg/asp/glu$^-$
6F3	*S. fradiae* No. 3535	met$^-$
6F3-1	*S. fradiae* No. 3535	met$^-$, arg$^-$
6F3-2	*S. fradiae* No. 3535	met$^-$, glu/asp/arg$^-$
6F3-3	*S. fradiae* No. 3535	met$^-$, thr$^-$
6F3-4	*S. fradiae* No. 3535	met$^-$, thr$^-$, SM^r
6F4	*S. fradiae* No. 3535	met$^-$
6F4-1	*S. fradiae* No. 3535	met$^-$, isl/leu$^-$
6F4-2	*S. fradiae* No. 3535	met$^-$, arg/lys$^-$
6F4-3	*S. fradiae* No. 3535	met$^-$, his/cys/glu$^-$
6F4-4	*S. fradiae* No. 3535	met$^-$, tyr$^-$
6F4-5	*S. fradiae* No. 3535	met$^-$, lys/arg$^-$
6F4-6	*S. fradiae* No. 3535	met$^-$, his$^-$
6F4-7	*S. fradiae* No. 3535	met$^-$, glu/arg$^-$
6F4-8	*S. fradiae* No. 3535	met$^-$, val$^-$
6F4-9	*S. fradiae* No. 3535	met$^-$, cys/glu$^-$
6F4-10	*S. fradiae* No. 3535	met$^-$, isl$^-$, SM^r
6F4-11	*S. fradiae* No. 3535	met$^-$, isl/leu$^-$, KY^r
6F4-12	*S. fradiae* No. 3535	met$^-$, isl/leu$^-$, CT^r
6F5	*S. fradiae* No. 3535	his$^-$
6F5-1	*S. fradiae* No. 3535	his$^-$, VM^r
6F5-2	*S. fradiae* No. 3535	his$^-$, NO^r
6F5-3	*S. fradiae* No. 3535	his$^-$, SM^r
6F5-4	*S. fradiae* No. 3535	his$^-$, met$^-$
6F5-5	*S. fradiae* No. 3535	his$^-$, asp/glu/arg$^-$
6F5-6	*S. fradiae* No. 3535	his$^-$, arg$^-$
6F5-7	*S. fradiae* No. 3535	his$^-$, (isl + val)$^-$,
6F5-8	*S. fradiae* No. 3535	his$^-$, met$^-$
6F5-9	*S. fradiae* No. 3535	his$^-$, cys$^-$
6F5-10	*S. fradiae* No. 3535	his$^-$, ser/gly/lys/thr
6F5-11	*S. fradiae* No. 3535	his$^-$, cys
6F5-12	*S. fradiae* No. 3535	his$^-$, met$^-$
6F5-13	*S. fradiae* No. 3535	his$^-$, arg$^-$
6F5-14	*S. fradiae* No. 3535	his$^-$, met$^-$
6F5-15	*S. fradiae* No. 3535	his$^-$, glu/asp/lys$^-$
6F5-16	*S. fradiae* No. 3535	his$^-$, arg$^-$, SM^r

TABLE 1 (*Continued*)

Code	Derived from strain	Markers*
6F5-17	*S. fradiae* No. 3535	his$^-$, arg$^-$, KY^r
6F7	*S. fradiae* No. 3535	cys/met$^-$
1G1	*S. griseoflavus* (H. Saito and Y. Ikeda)	ade$^-$, met$^-$
1G2	*S. griseoflavus* (H. Saito and Y. Ikeda)	gly$^-$, try$^-$
1G3	*S. griseoflavus* (H. Saito and Y. Ikeda)	gly$^-$, cys/nia$^-$
4B17	*S. griseus* No. 3475	arg$^-$
4B18	*S. griseus* No. 3475	(isl + val)$^-$
4B19	*S. griseus* No. 3475	arg$^-$
4B19-1	*S. griseus* No. 3475	arg$^-$, met$^-$
4B19-2	*S. griseus* No. 3475	arg$^-$, his$^-$
4B20	*S. griseus* No. 3475	his$^-$
4B21	*S. griseus* No. 3475	his$^-$
4B23	*S. griseus* No. 3475	thr/pro/gly/ser$^-$
4B24	*S. griseus* No. 3475	his$^-$
4B24-1	*S. griseus* No. 3475	his$^-$, TR^r
4B24-2	*S. griseus* No. 3475	his$^-$, CI^r
4B24-3	*S. griseus* No. 3475	his$^-$, ser/gly/thr$^-$
4B24-4	*S. griseus* No. 3475	his$^-$, met$^-$
4B24-5	*S. griseus* No. 3475	his$^-$, gly/ser$^-$
4B24-6	*S. griseus* No. 3475	his$^-$, CT^r
4B24-7	*S. griseus* No. 3475	his$^-$, cys/arg/met$^-$, Ri
4B24-8	*S. griseus* No. 3475	his$^-$, MY^r
4B24-9	*S. griseus* No. 3475	his$^-$, TE^r
4B24-10	*S. griseus* No. 3475	his$^-$, CA^r
4B24-11	*S. griseus* No. 3475	his$^-$, CT^r
4B24-12	*S. griseus* No. 3475	his$^-$, ser/gly$^-$, NO^r
4B24-13	*S. griseus* No. 3475	his$^-$, ser/gly$^-$, CT^r
3B1	*S. griseus* No. 3586	his$^-$
3B2	*S. griseus* No. 3586	arg$^-$
3B3	*S. griseus* No. 3586	his$^-$
3B4	*S. griseus* No. 3586	lys$^-$
3B4-1	*S. griseus* No. 3586	lys$^-$, leu$^-$
3B4-2	*S. griseus* No. 3586	lys$^-$, ade/xan/gua$^-$
3B4-3	*S. griseus* No. 3586	lys$^-$, ade$^-$
3B4-4	*S. griseus* No. 3586	lys$^-$, ade$^-$
3B4-5	*S. griseus* No. 3586	lys$^-$, ade$^-$
3B5	*S. griseus* No. 3586	his$^-$
3B6	*S. griseus* No. 3586	pro$^-$
3B6-1	*S. griseus* No. 3586	pro$^-$, arg$^-$
3B6-2	*S. griseus* No. 3586	pro$^-$, met$^-$
3B6-3	*S. griseus* No. 3586	pro$^-$, arg$^-$
3B6-4	*S. griseus* No. 3586	pro$^-$, leu$^-$
3B6-5	*S. griseus* No. 3586	pro$^-$, his$^-$
3B6-6	*S. griseus* No. 3586	pro$^-$, thr$^-$
3B6-7	*S. griseus* No. 3586	pro$^-$, (isl + val)$^-$
3B6-8	*S. griseus* No. 3586	pro$^-$, his$^-$
3B6-9	*S. griseus* No. 3586	pro,$^-$ arg$^-$
3B6-10	*S. griseus* No. 3586	pro$^-$, arg$^-$
3B6-11	*S. griseus* No. 3586	pro$^-$, his$^-$
3B6-12	*S. griseus* No. 3856	pro$^-$, arg$^-$
3B6-13	*S. griseus* No. 3586	pro$^-$, thr$^-$, SM^r
3B7	*S. griseus* No. 3586	(isl + val)$^-$
3B8	*S. griseus* No. 3586	his$^-$
3B9	*S. griseus* No. 3586	arg$^-$
3B10	*S. griseus* No. 3586	his$^-$
3B11	*S. griseus* No. 3586	his$^-$

TABLE 1 (*Continued*)

Code	Derived from strain	Markers*
3B12	*S. griseus* No. 3586	ser/gly$^-$
2C9	*S. parvus* No. 3686	cys/met$^-$
2C9-1	*S. parvus* No. 3686	cys/met$^-$, SM^r
2C9-2	*S. parvus* No. 3686	cys/met$^-$, VI^r
2C9-3	*S. parvus* No. 3686	cys/met$^-$, CT^r
2C10	*S. parvus* No. 3686	his$^-$
2C11	*S. parvus* No. 3686	arg$^-$
2C12	*S. parvus* No. 3686	cys/met$^-$
2a870	*S. rimosus* (S. I. Alikhanian)	his$^-$
8229-310	*S. rimosus* (S. I. Alikhanian)	(isl + val)$^-$
2E2	*S. spheroides*	met/cys$^-$
2E3	*S. spheroides*	his$^-$
2E4	*S. spheroides*	his$^-$
7A3	*S. venezuelae* No. 3625	ade/xan/gua$^-$
7A4	*S. venezuelae* No. 3625	his$^-$
7A25	*S. venezuelae* No. 3625	(isl + val)$^-$
7A26	*S. venezuelae* No. 3625	arg/glu/asp$^-$
7A32	*S. venezuelae* No. 3625	undetermined

* Symbols for nutritional requirements: ade$^-$, adenine; arg$^-$, arginine; asp$^-$, aspartic acid; cys$^-$, cystine; glu$^-$, glutamic acid; gua$^-$, guanine; gly$^-$, glycine; his$^-$, histidine; isl$^-$, isoleucine; leu$^-$, leucine; lys$^-$, lysine; met$^-$ methionine; nia$^-$, niacin; pha$^-$, phenylalanine; pro$^-$, proline; ser$^-$, serine; thr$^-$, threonine; tyr$^-$, tyrosine; val$^-$, valine; xan$^-$, xanthine; CA^r, carbomycin; resistance; CT^r, catenulin resistance; CI^r, cinnamycin resistance; KY^r, kyostacin resistance; MY^r, mycomycetin resistance; NO^r, novobiocin resistance; SM^r, streptomycin resistance; TE^r, tetracycline resistance; TY^r, tyrocidin resistance; VI^r, viomycin resistance; pigm$^+$, pigment production; Ri, morphological mutant.

Establishment of Heterokaryosis

When two different strains of *Streptomyces* are cultivated together they may interact in several ways. Interaction at a distance without physical contact between the two types of hyphae is exemplified by antibiosis and syntrophy, in which diffusible substances produced by one strain inhibit or promote, respectively, the growth of another strain. Direct contact between mycelia may lead to another type of interaction, anastomosis, or fusion of hyphae. When conditions are favorable, the result is the establishment of heterokaryotic growth, which means that nuclei of the two parental types, although still discrete, intact units, become and remain evenly intermixed in the same cytoplasmic field within a common hypha (Lederberg, 1955). The nuclear diversity in a heterokaryotic mycelium need not necessarily originate from the union of separate parental strains, since a new type of nucleus could also arise by mutation in a previously homokaryotic strain.

Heterokaryosis was observed originally in filamentous fungi (for earlier references, see Pontecorvo, 1946), while the occurrence of this phenomenon in *Streptomyces* first was mentioned briefly by Lederberg (1955) and later by Sermonti and Spada-Sermonti (1955). More extensive evidence for heterokaryosis in this group of organisms was presented almost simultaneously by Szybalski and Braendle (1956), and Bradley and Lederberg (1956).

FIGURE 1 illustrates the principle of heterokaryon formation. To demonstrate this phenomenon in an unequivocal manner, however, it was necessary to employ nutritionally marked mutant strains. Filtered conidial suspensions

of *S. fradiae* strains 6F4-1 and 6F5-6, prepared in the manner described in the previous section, were plated both separately (controls) and as a mixture on minimal agar. Strain 6F4-1 required methionine and isoleucine or valine for growth, while mutant 6F5-6 was histidine- and arginine-dependent. Colonies appeared only on the plates seeded with the mixture of conidia. In order to prove that nutritional cooperation had been established between diverse nuclei in the same hypha, rather than between nuclei of unlike homokaryotic hyphae,

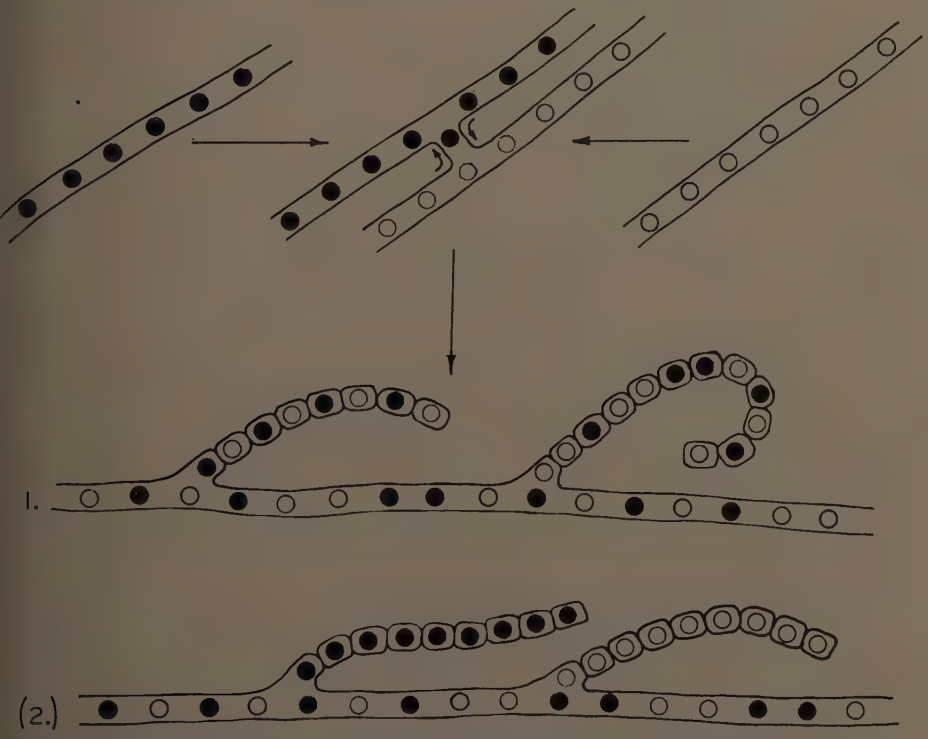

FIGURE 1. Diagrammatic representation of heterokaryon formation. Fusion of two hyphae containing different types (black and white) of nuclei produces a heterokaryotic mycelium with well-intermixed nuclei. Conidial chains produced by the heterokaryon must be either (1) heterokaryotic (as confirmed experimentally, see text) or (2) homokaryotic.

it was necessary to isolate by micromanipulation intact pieces of the putative heterokaryotic mycelium and to demonstrate their ability to grow under selective, prototrophic conditions. The second criterion for heterokaryosis, as generally defined (Lederberg, 1955), is the recovery of both parental combinations of auxotrophic properties in all uninuclear derivatives of the heterokaryon, the conidia. Heterokaryotic growth thus cannot be propagated through uninuclear conidia, which predominate in *Streptomyces* (Braendle and Szybalski, 1957; Kinoshita and Itagaki, 1958; Saito and Ikeda, elsewhere in this monograph). Even in the case of bi- or trinuclear spores, there is a high and theoretically predictable proportion of parental segregants (Atwood and Mukai,

1955). The preservation of the heterokaryotic state indeed must necessitate an efficient mechanism of intramycelial mixing of nuclei. In view of the less than 1 μ lumen of the hyphae, the establishment of two-way traffic would present formidable difficulties. Should the nuclear intermixing not function effectively, groups of identical nuclei would accumulate in long hyphal stretches and heterokaryosis could be restored only by frequent anastomoses between segments containing unlike nuclei.

From the above considerations, it may be implied that a balanced heterokaryon is preserved only under strongly selective conditions. The propagation of a heterokaryon through uninuclear conidia is impossible, by definition. Half of the binuclear conidia would be dikaryotic (containing exclusively pairs of diverse nuclei) if the nuclei in the sporogenic mycelium were perfectly intermixed. The only simple mechanism that could lead to the exclusive formation of dikaryotic (cooperative) conidia, and thus to failure to yield parental segregants, would involve some inherent inability of both parental strains to form homokaryotic conidia. This condition, however, has not hitherto been demonstrated, and probably could not be involved in the case described as heterokaryosis in *S. coelicolor* by Bradley (1957, 1958), since each parental strain was able to form conidia.

It has been observed that not all hyphal fragments derived from nutritionally balanced heterokaryons are able to propagate on minimal agar (Bradley and Lederberg, 1956). These deficient fragments may grow on partially supplemented agar that, however, does not support the growth of either parent. Similarly, we have observed the production of heterokaryotic colonies from doubly auxotrophic parents on agar supplemented with only one growth factor (Braendle and Szybalski, 1957). These colonies segregated both the parental types of conidia, but small fragments when transferred to unsupplemented minimal agar did not give rise to prototrophic growth. This situation, described as anomalous heterokaryosis by Bradley and Lederberg (1956) or nutritionally unbalanced heterokaryosis by Braendle and Szybalski (1957), is illustrated in FIGURE 2. The partial requirement of methionine in one case, and arginine in the other case, is hypothesized to originate from a shift in the relative numbers of parental nuclei. The partial deficiency indicates a scarcity of nuclei controlling the production of a given growth factor, especially if the inherent capability of the producer nucleus to govern the particular synthesis is already under par. The working hypothesis outlined in FIGURE 2 seems to be the most plausible explanation of the observed phenomenon. The hypothesis was tested by determining the nuclear ratios as represented by the relative frequencies of the two types of parental conidia derived from nutritionally balanced and unbalanced heterokaryons. When the unbalanced heterokaryons exhibited single deficiencies (histidine or arginine, FIGURE 2), the conidial ratio was shifted in the predicted direction, in relation to that of the balanced heterokaryon, by over a tenfold increment. However, no large differences in the conidial ratio were detectable between balanced and unbalanced heterokaryons when the latter exhibited two nonparental deficiencies (histidine and arginine, FIGURE 2). The explanation for this discrepancy may lie in inadequate intermixing of the two types of cooperating nuclei and resulting

poor reciprocal exchange of nutrients throughout the mycelium, as represented at the bottom of FIGURE 2.

Antibiotic Resistance and Antibiotic Production in Heterokaryons

Heterokaryosis provides a means for determining the relative dominance or recessiveness of allelic characters, even in the absence of a stable diploid

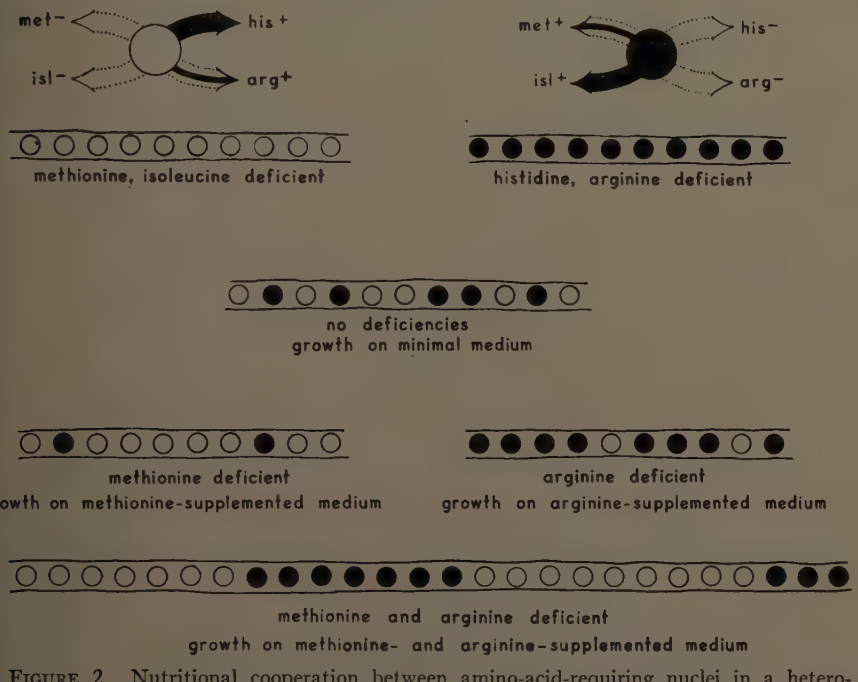

FIGURE 2. Nutritional cooperation between amino-acid-requiring nuclei in a heterokaryon. White nuclei control the production of only sufficient amounts of arginine (*thin arrow*) and an excess of histidine (*thick arrow*), while an external supply of methionine and isoleucine is required (*upper left*). Black nuclei control the production of only sufficient amounts of methionine (*thin arrow*) and an excess of isoleucine (*thick arrow*), while an external supply of histidine and arginine is required (*upper right*). In the heterokaryon with well-intermixed nuclei (*center*), no nutritional deficiencies are observed. A shift in the nuclear ratio toward scarcity of black (*lower left*) or white (*lower right*) nuclei results in a methionine or arginine requirement, respectively, of the resulting unbalanced heterokaryon. Defective intermixing of nuclei (*lower drawing*) produces arginine and methionine deficiencies.

phase. In previous studies all of the nutritional requirements under investigation behaved as recessive alleles of nutritional independence, since a heterokaryon synthesized from, for example, histidine-deficient and histidine-independent mutants was of prototrophic character. Subsequently, the behavior of streptomycin or catenulin-resistance markers was studied in prototrophic heterokaryons synthesized from nutritionally deficient sensitive and resistant strains on minimal agar. Small amounts of sporefree mycelium from such heterokaryons were transferred to fresh minimal agar with and without antibiotics. Failure of growth to appear on the streptomycin-containing medium

(TABLE 2) constituted evidence for the recessive character of the resistance markers. In other words, the presence of resistant nuclei in the heterokaryons did not protect the whole organism from the inhibitory action of the antibiotic in question. The discovery of a highly selective, dominant resistance marker would greatly facilitate the rapid survey of unmarked wild-type strains for heterokaryosis. A nutritionally deficient strain with a dominant resistance marker could serve as the tester stock, since only heterokaryotic recombinants produced in crosses between this stock and antibiotic-sensitive, prototrophic

TABLE 2

ANTIBIOTIC SENSITIVITY OF HETEROKARYONS DERIVED FROM ANTIBIOTIC-SENSITIVE AND -RESISTANT PARENTAL STRAINS

Antibiotic	Parental strains			Inhibitory concentrations (μg./ml.)	
	Species	Code	Markers*	Parental	Heterokaryon
Streptomycin	*S. fradiae*	6F4-1	met⁻, isl⁻, SM_1^r	>100	2
		6F5-16	his⁻, arg⁻	2	
Streptomycin	*S. fradiae*	6F4-10	met⁻, isl⁻	2	2
		6F5-13	his⁻, arg⁻, SM_2^r	>100	
Streptomycin	*S. fradiae*	6F4-10	met⁻, isl⁻	2	2
		6F5-13a	his⁻, arg⁻, SM_3^r	>100	
Streptomycin	*S. fradiae*	6F4-10	met⁻, isl⁻	2	2
		6F5-13b	his⁻, arg⁻, SM_4^r	>100	
Streptomycin	*S. fradiae*	6F4-1	met⁻, isl⁻, SM_1^r	>100	>100
		6F5-13	his⁻, arg⁻, SM_2^r	>100	
Catenulin	*S. griseus*	4B24-6	his⁻, CT^r	40	1.5
		4B26	cys/met⁻	1.5	
Catenulin	*S. griseus*	4B24	his⁻	1.5	1.5
		4B26-2	cys/met⁻, CT^r	>100	
Catenulin	*S. griseus*	4B24-13	his⁻, ser/gly⁻, CT^r	>100	1.5
		4B19-1	arg⁻, met⁻	1.5	

* For symbols and abbreviations, see TABLE 1.

strains can grow on antibiotic-containing minimal agar. A roughly analogous system was devised by Lederberg (1951) for surveying recombination in bacteria. Unfortunately, the search for dominant resistance markers hitherto has been unsuccessful.

We have also evaluated in heterokaryons the behavior of another genetic property: the capacity to produce antibiotics. The elaboration of neomycin by *S. fradiae* was selected as a model system. The wild-type strain 3535 is a neomycin producer. This property was not affected in the nutritionally marked mutants of this strain, with only one exception (TABLE 3). It therefore was necessary to screen for more mutants that had lost the capacity to produce neomycin. A number of the marked strains of *S. fradiae* (TABLE 1) were ir-

radiated by ultraviolet to the 1 to 50 per cent survival level and plated on nutrient agar at dilutions such that 20 to 30 colonies/plate appeared. The plates were incubated for 4 days, then overlayered with 3 ml. of 0.6 per cent agar heavily inoculated with spores of *Bacillus subtilis*. After overnight incubation, the colonies were inspected for inhibition zones. This screening procedure, however, failed to select any nonproducing mutants, thus demonstrating that the capacity to produce neomycin is a very stable property.

Having failed in the search for a nonneomycin-producing mutant of *S. fradiae*, we selected for use the histidine- and arginine-deficient strain 6F5-8, which was a very late and poor producer (TABLE 3). This strain was crossed to neomycin-producing, isoleucine- and methionine-requiring strain 6F4-1 on minimal agar. A second cross was made between strain 6F4-1 and histidine-

TABLE 3

NEOMYCIN PRODUCTION BY NUTRITIONAL MUTANTS OF *S. FRADIAE* NO. 3535

Strain*	Inhibition zone, mm.†	Strain*	Inhibition zone, mm.†
3535	10	6F4-9	7
6F2	11	6F4-10	7
6F3	8	6F4-11	7
6F3-1	8	6F5	10
6F3-2	9	6F5-1	7
6F3-3	8	6F5-2	9
6F4	6	6F5-3	6
6F4-1	10	6F5-4	10
6F4-2	8	6F5-5	7
6F4-3	7	6F5-6	7
6F4-4	8	6F5-7	9
6F4-5	3	6F5-8	0–1
6F4-6	3	6F5-11	7
6F4-7	6	6F5-16	8
6F4-8	5	6F7	10

* For description of strains, see TABLE 1.

† Inhibition zone around 4-day-old colonies overlayered by 0.6 per cent agar seeded with *B. subtilis* spores.

and arginine-requiring, neomycin-producing strain 6F5-13 on minimal agar. The neomycin production of the resulting nutritionally balanced heterokaryons was compared with that of the wild-type strain on minimal agar. Other selective media were also employed, as listed in TABLE 4. A semiquantitative method of neomycin assay, based on measurement of the inhibition zone produced by colonies of the same age, was applied throughout this experiment. The high stability of this antibiotic made the assay fairly reliable, as indicated by the reproducibility of the results. The procedure involved plucking out selected whole colonies grown on minimal or supplemented minimal agar to equal size, removal of adhering agar fragments, and transfer to the surface of the assay media listed in TABLE 4. The plates were flooded with *Bacillus subtilis*-seeded, 0.6 per cent agar after 1, 2, or 4 days' incubation. It is apparent from TABLE 4 that neomycin production behaved as a dominant character in this experiment. The amounts of antibiotic elaborated by the wild-type strain and by heterokaryons composed of one or both producing components were

quite comparable. Neomycin production on minimal medium containing glucose and NH_4^+ ions as the sole carbon and nitrogen sources compared favorably with the yield obtained on complex nutrient media. The yield of mutants on nutrient agar was almost identical with that of the wild-type strain; however, on minimal agar supplemented with the required amino acids (at levels of 10 μg./ml.), the yields of strains 6F4-1 and 6F5-13 were much lower, even though growth was supported. Moreover, heterokaryons that readily produced neomycin either on minimal medium or nutrient agar were inefficient producers on amino acid-supplemented media. It is possible that in hetero-

TABLE 4

NEOMYCIN PRODUCTION BY HETEROKARYONS OF *S. FRADIAE*

Medium*	Incubation period	Strains†				Heterokaryons	
		3535 wild	6F4-1 met⁻, isl⁻	65F-13 his⁻, arg⁻	6F5-8 his⁻, arg⁻	6F4-1 6F5-13	6F4-1 6F5-8
NA	1 day	19‡	16	19	0	15	15
WMA		20				15	12
WMA + met + his		12				12	11
WMA + leu + his + met + his		11				6	N.G.
WMA + leu + arg		10	7	8	0	0	0
NA	2 days	32	29	30	0	23	30
WMA		27				16	16
WMA + met + his		30				15	14
WMA + leu + his + met + his		29				13	N.G.
WMA + leu + arg		27	8	8	0	0	—
NA	4 days	>50	>50	>50	0	>50	>50
WMA		50				36	32
WMA + met + his		46				24	30
WMA + leu + his + met + his		35				16	0
WMA + leu + arg		40	10	15	4	14	0

* Key: NA, nutrient agar; WMA, minimal agar prepared from agar washed exhaustively with water and subsequently freeze-dried; for other abbreviations, see TABLE 1.

† For symbols, see TABLE 1. N.G.: no growth.

‡ Inhibition zones (mm.) around 1- to 4-day-old colonies; see TABLE 3.

karyons formed on the latter media the parental nuclei were not intermixed sufficiently to permit optimal metabolic cooperation, with the result that neomycin production was even lower than that of the parental strains. However, the possibility of an inhibitory effect for one of the amino acids cannot be excluded, especially since even the wild-type strain produced lower yields on supplemented than on nonsupplemented minimal agar. The complex nutritional effect of amino acids and other growth factors on antibiotic production by artificially produced, nutritionally deficient strains is wide open for further study.

Mechanism of Conidia Formation in S. fradiae

Direct observations of conidia formation in *Streptomyces* (Waksman, 1950) and electron micrographs of the conidial chains (FIGURE 3) indicate that these

uninuclear units are produced by simple fragmentation of aerial hyphae. The mechanism is therefore different from that observed for fungi such as, for example, *Aspergillus* or *Penicillium*. In the latter, a specialized cell (secondary sterigmatum) gives rise to a chain of conidia, the nuclei of which are all derived from a common ancestor, so that in heterokaryons the conidia of a given chain are of one parental type only. In heterokaryons of *Streptomyces*, on the other hand, each conidial chain should contain both parental types, provided the nuclei in the sporogenous hyphae are well intermixed.

FIGURE 3. Electron micrograph of a conidial chain of *S. rimosus* grown on Bennett's agar medium. Micrograph prepared by Miss P. E. Holbert.

The alternative possibilities, (1) heterokaryotic and (2) homokaryotic conidial chains, are depicted in FIGURE 1, and the principle of a method for differentiating them is outlined in FIGURE 4. Two strains of *S. fradiae* were selected, one requiring histidine and the other cysteine, and both producing conidia with strongly impaired capacity to germinate on minimal medium in the absence of the required amino acid. The germination of conidial chains derived from heterokaryons was followed by microscopic observation, initially on singly and subsequently on doubly supplemented media. By this means, both parental types were shown to be present in a single chain, although disruption of the chains caused by their movement during germination made interpretation somewhat difficult. These observations favor the view that the mechanism of conidia production involves simple fragmentation of the aerial heterokaryotic hyphae into uninuclear spores. Heterokaryotic conidial chains therefore should be the rule. However, if long stretches of aerial hyphae, although

derived from a heterokaryon, contained only one type of nucleus, chains of identical conidia would be expected to predominate.*

Taxonomic Implications of Heterokaryosis

The frequency of prototrophic heterokaryon formation on minimal agar plates seeded with approximately 10^8 conidia of each parental auxotrophic strain was highly variable, ranging from a few to many thousand colonies per plate. This was true for obligatory deficient mutants which, when seeded

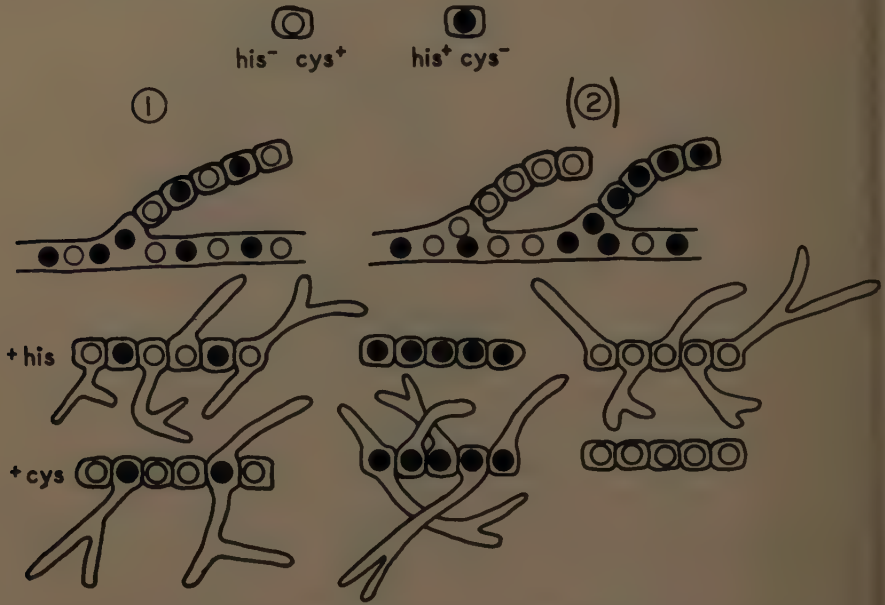

FIGURE 4. The principle behind the experimental determination of conidial distribution in chains. (1) A portion of chains containing both types of conidia should germinate on histidine-supplemented minimal medium, and the remainder of the conidia should germinate when the second supplement, cysteine, is added. This situation and the reverse were confirmed by microscopic observation (see text). (2) Homogeneity of the conidial chain should be reflected in failure of the entire chain to germinate on one medium, and in complete germination on another. Deficiencies that interfere with conidial germination were selected for this experiment.

alone on minimal agar, did not exhibit perceptible residual growth and were not syntrophic in the combination used. Obviously, in the case of "leaky" mutants (showing pronounced residual growth), or if syntrophic interaction occurred, the apparent frequency of heterokaryon formation could be much higher, as observed by Bradley and Lederberg (1956).

The formation of heterokaryons was observed to occur only between mutants derived from the same species. Heterokaryosis was demonstrated for *S. griseus*, *S. fradiae*, *S. albus*, *S. venezuelae*, *S. griseoflavus*, *and S. spheroides*. No nutritionally balanced heterokaryons were formed between mutants of *S.*

* This situation was apparently encountered by Bradley *et al.*, as reported elsewhere in this monograph.

parvus, although the experiments with this species were very limited in scope. Similarly, nutritionally balanced heterokaryosis was not observed for *S. coelicolor* mutants under the experimental conditions employed in this study, despite the fact that synkaryosis was demonstrated for this species, as discussed below. TABLE 5 is based on our previous studies (Braendle and Szybalski, 1957) supplemented with additional data on the occurrence of heterokaryosis within different species of *Streptomyces*.

Coparticipation in heterokaryosis would seem to indicate a very close relationship between two unclassified strains, which on this basis, if so defined, could be placed in the same species. On the other hand, the reverse need not necessarily hold true, because failure of heterokaryosis was observed for some combinations of mutants derived from the same species (for example, strains 4B24 and 4B22 of *S. griseus*, TABLE 5). The occurrence of heterokaryosis between *S. griseus* and *S. cyaneus*, as reported by Bradley and Lederberg (1956), might reflect a close taxonomic relationship between these two species or possibly some peculiarity of the heterokaryotic assay system. With the development of suitable tester stocks bearing a dominant resistance marker in addition to auxotrophic deficiencies, as discussed in the previous section, the assay of heterokaryotic interaction could become a taxonomic tool of primary importance for this economically important genus.

Heterokaryotic Compatibility

A heterokaryotic compatibility system in *S. fradiae* has been characterized. Since the observations have been described recently in detail (Braendle *et al.*, 1959), they will only be summarized here. The following illustration will serve to describe the observed phenomenon, which is also schematically represented in FIGURE 5. When 10^8 conidia of each of two freshly isolated auxotrophic mutants, 6F4-1 and 6F5-16, were plated on minimal agar, a small number of heterokaryotic colonies, usually 1 to 5, appeared on each plate. These original parental strains were designated as low-frequency mating (LFM) strains. From the conidia produced by these heterokaryotic colonies, new parental lines were derived and designated as secondary parental isolates. The frequency of heterokaryosis between these newly isolated lines was several thousand times higher than between the original parental strains. The secondary parental isolates were designated as high-frequency mating (HFM) strains.

The nature of the difference between the LFM and HFM strains was examined experimentally as follows. First it was determined that both of the HFM isolates possessed a new property. Only crosses between the two HFM strains resulted in a heterokaryotic mating frequency in the neighborhood of 5000 (the ratio of the number of heterokaryotic colonies to the number of conidia of the minority parent in the inoculum $\times 10^8$). The heterokaryotic mating frequency for crosses between two LFM parents was less than 1, and between one LFM and one HFM parent it ranged from 5 to 90.

The HFM property was found to be reasonably stable, remaining undiminished for 22 serial transfers (on agar slants) in one experiment, and returning to low mating frequency only after 12 transfers in another case. That the HFM property is of heritable nature is suggested on the one hand by its high stability and, on the other hand, by the unpredictable, stochastic nature of its

TABLE 5
OCCURRENCE OF HETEROKARYOSIS AND SYNKARYOSIS IN STREPTOMYCES

Parent I			Parent II			Genetic interaction†	
Species	Mutant*	Markers*	Species	Mutant*	Markers*	Hetero-karyosis	Syn-karyosis
S. albus 618	3E12	met⁻	*S. albus* 618	3E13	not detn.	+	(−)
S. coelicolor (Sermonti)	14D1	met⁻, his⁻	*S. coelicolor* (Sermonti)	14D2	pro⁻, glu⁻	−	+
S. coelicolor (Sermonti)	14D1-2	met⁻, his⁻, SM^r	*S. coelicolor* (Sermonti)	14D2	pro⁻, glu⁻	−	+
S. coelicolor (N.I. 9021)	13D3	met⁻	*S. coelicolor* (N.I. 9021)	13D4	arg⁻	−	+
S. coelicolor (N.I. 9021)	13D3	met⁻	*S. coelicolor* (Sermonti)	14D2	pro⁻glu⁻	−	+
S. coelicolor (Hopwood)	12D1	his⁻	*S. coelicolor* (Hopwood)	12D2	met⁻	−	+
S. coelicolor (Hopwood)	12D1	his⁻	*S. coelicolor* (N.I. 9021)	13D4	arg⁻	−	−
S. coelicolor (Hopwood)	12D2	met⁻	*S. coelicolor* (N.I. 9021)	13D4	arg⁻	−	−
S. coelicolor (Hopwood)	12D2	met⁻	*S. coelicolor* (N.I. 9021)	13D3	met⁻	−	+
S. coelicolor (Hopwood)	12D2	met⁻	*S. coelicolor* (Sermonti)	14D2	pro⁻glu⁻	−	−
S. coelicolor (Hopwood)	12D4	met⁻, pha⁻	*S. coelicolor* (Sermonti)	14D2	pro⁻glu⁻	−	−
S. fradiae 3535	6F5	his⁻	*S. fradiae* 3535	6F7	cys/met⁻	+	
S. fradiae 3535	6F5	his⁻	*S. fradiae* 3535	6F4	met⁻	+	
S. fradiae 3535	6F5	his⁻	*S. fradiae* 3535	6F2	arg/asp/glu⁻	+	
S. fradiae 3535	6F4	met⁻	*S. fradiae* 3535	6F7	cys/met⁻	+	
S. fradiae 3535	6F4-1	met⁻, isl/val⁻	*S. fradiae* 3535	6F5-6	his⁻, arg⁻	+	
S. fradiae 3535	6F4-1	met, isl/val⁻	*S. fradiae* 3535	6F5-9	his⁻, cys⁻	+	
S. fradiae 3535	6F4-2	met⁻, arg/lys⁻	*S. fradiae* 3535	6F5-7	his⁻, val + isl⁻	+	
S. fradiae 3535	6F4-2	met⁻, arg/lys⁻	*S. fradiae* 3535	6F5-9	his⁻, cys⁻,	+	
S. fradiae 3535	6F3-3	met⁻, thr⁻	*S. fradiae* 3535	6F5-16	his⁻, arg⁻, SM^r	+	+
S. fradiae 3535	6F3-3	met⁻, thr⁻	*S. fradiae* 3535	6F5-7	his⁻, val + isl⁻	+	+
S. fradiae 3535	6F4-1	met⁻, isl/val⁻	*S. fradiae* 3535	6F5-16	his⁻, arg⁻SM^r	+	+
S. fradiae 3535	6F4-10	met⁻, isl/val⁻SM^r	*S. fradiae* 3535	6F5-13	his⁻, arg⁻	+	+
S. griseoflavus (Saito)	1G1	ade⁻met⁻	*S. griseoflavus* (Saito)	1G1	gly⁻try⁻	+	+
S. griseus 3475	4B24	his⁻	*S. griseus* 3475	4B26	cys/met⁻	+	−
S. griseus 3475	4B24	his⁻	*S. griseus* 3475	4B22	cys/met⁻	−	−
S. griseus 3475	4B24-5	his⁻, ser/gly⁻	*S. griseus* 3475	4B18	val + isl⁻	+	+
S. griseus 3475	4B24	his⁻	*S. griseus* 3475	4B20	his⁻	−	−
S. griseus 3475	4B26	cys/met⁻	*S. griseus* 3475	4B20	his⁻	+	−
S. griseus 3475	4B26	cys/met⁻	*S. griseus* 3475	4B18	val + isl⁻	+	−

S. griseus 3475	4B26	cys/met⁻	*S. griseus* 3475	4B17	arg⁻	+	−
S. griseus 3475	4B26	cys/met⁻	*S. griseus* 3475	4B21	his⁻	+	−
S. griseus 3475	4B26	cys/met⁻	*S. griseus* 3475	4B24-5	his⁻, ser/gly⁻	+	−
S. griseus 3475	4B26	cys/met⁻	*S. griseus* 3475	4B24-3	his⁻, ser/gly/thr⁻	+	−
S. griseus 3475	4B19-1	arg⁻met⁻	*S. griseus* 3475	4B24-13	his⁻, ser/gly⁻, CTr	+	+
S. griseus 3475	4B24-4	his⁻met⁻	*S. griseus* 3586	3B6-4	pro⁻, leu⁻	+	−
S. griseus 3475	4B19-1	arg⁻met⁻	*S. griseus* 3586	3B6-6	pro⁻, thr⁻	−	−
S. griseus 3586	3B1	his⁻	*S. griseus* 3586	3B6-4	pro⁻, leu⁻	+	−
S. griseus 3586	3B1	his⁻	*S. griseus* 3586	3B9	his⁻	−	−
S. griseus 3586	3B1	his⁻	*S. griseus* 3586	3B6	pro⁻	−	+
S. griseus 3586	3B1	his⁻	*S. griseus* 3586	3B7	isl + val⁻	+	−
S. griseus 3586	3B5	his⁻	*S. griseus* 3586	3B6-6	pro⁻, thr⁻	−	−
S. parvus 3686	2C11	arg⁻	*S. parvus* 3686	2610	his⁻	−	−
S. parvus 3686	2C11	arg⁻	*S. parvus* 3686	2C9-1	cys/met⁻SMr	−	−
S. spheroides	2E1	his⁻	*S. spheroides*	2F2	cys/met⁻	+	(−)
S. venezuelae 3625	7A3	tyr⁻	*S. venezuelae* 3625	7A26	arg/asp/glu⁻	−	−
S. venezuelae 3625	7A4	arg⁻	*S. venezuelae* 3625	7A32	not detn.	+	(−)
S. venezuelae 3625	7A4	arg⁻	*S. venezuelae* 3625	7A26	arg/asp/glu⁻	−	−

Inter-specific crosses

S. albus 618	3E12	met⁻	*S. spheroides*	2E1	his⁻	−	−
S. coelicolor (Sermonti)	14D1	his⁻met⁻	*S. griseus* 3475	4B24	his⁻	−	−
S. fradiae 3535	6F7	cys⁻/met⁻	*S. griseus* 3475	4B24	his⁻	−	−
S. griseoflavus (Saito)	1G2	gly⁻, try⁻	*S. coelicolor* (Sermonti)	14D2	pro⁻, glu⁻	−	−
S. griseus 3475	4B24	his⁻	*S. venezuelae* 3625	7A4	arg⁻	−	−
S. parvus 3686	2C9	cys/met⁻	*S. griseus* 3475	4B24	his⁻	−	−
S. spheroides	2E2	cys/met⁻	*S. albus* 618	3E13	not detn.	−	−
S. venezuelae 3625	7A4	arg⁻	*S. coelicolor* (Sermonti)	14D2	pro⁻, glu⁻	−	−

* For symbols and abbreviations, see TABLE 1.

† + denotes the occurrence of heterokaryosis or synkaryosis, and − denotes their absence. Parentheses indicate that the number of experiments was limited, while a blank denotes that no observations were made.

disappearance upon serial subculturing. FIGURE 6 illustrates hypothetically the automatic nature of selection of HFM mutant conidia assuming that heterokaryons can be formed only, or with much higher probability, between germinated HFM conidia. The opposite phenomenon, that is, the decrease in the mating frequency observed in one instance after the tenth to twelfth transfer, could be interpreted as associated with the emergence of an LFM mutant endowed with a selective advantage over the HFM strain. The loss of the HFM property by one partner resulted only in a precipitous drop in the heterokaryotic mating frequency, as discussed earlier in this section. The data thus indicate that the HFM property is not due to transient mixing of cytoplasm in

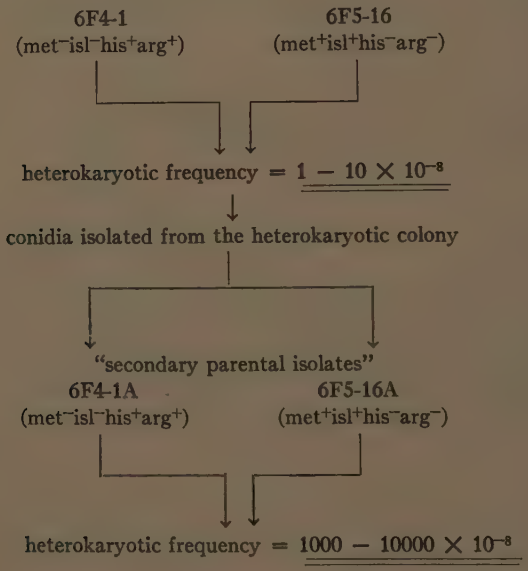

FIGURE 5. Heterokaryotic compatibility; see text.

conidia derived from heterokaryotic mycelia, but is of more stable heritable nature.

In further experiments, the possibility that the increase in mating frequency might be dependent on a greater amount of residual growth by the HFM strains was tested. As illustrated in FIGURE 7, the chance of encounter between auxotrophic conidia on selective media could be increased if longer germination tubes were produced before the cessation of growth. The extent of germination of LFM and HFM conidia, individually and in combinations, was evaluated quantitatively under the microscope (Braendle *et al.*, 1959). Since no appreciable differences between the HFM and LFM conidia in this respect were detected, the results failed to support the mechanism described in FIGURE 7. By elimination, therefore, it may be concluded that the HFM property depends on more effective hyphal fusion or intermixing of nuclei.

Interspecific heterokaryotic crosses were attempted between HFM auxotrophic mutants of *S. fradiae* and three other species (*S. venezuelae*, *S. griseus*, and *S. coelicolor*). The interspecific mating barrier apparently was not affected

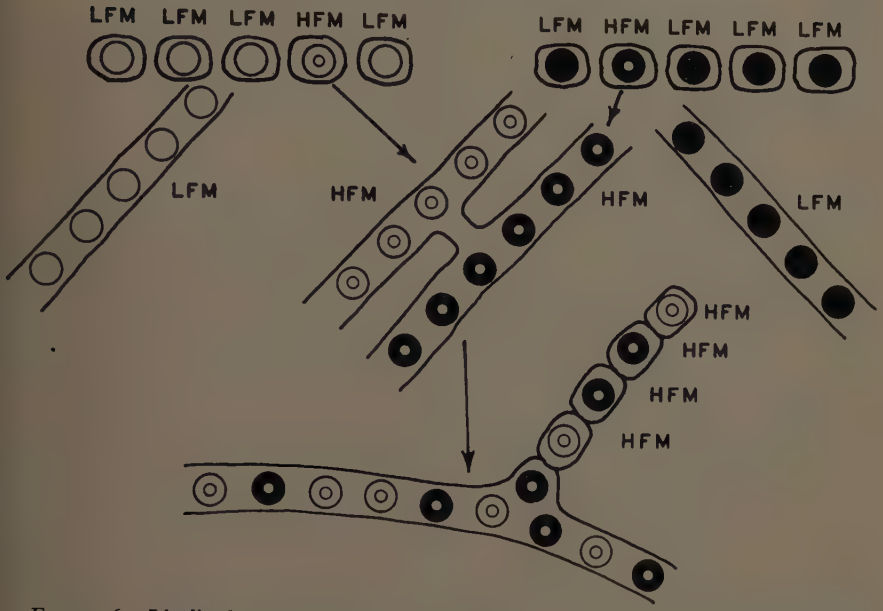

FIGURE 6. Idealized representation of the automatic selection of HFM (high-frequency mating) nuclei (represented by the two concentric circles) during formation of heterokaryons between LFM (low-frequency mating) strains.

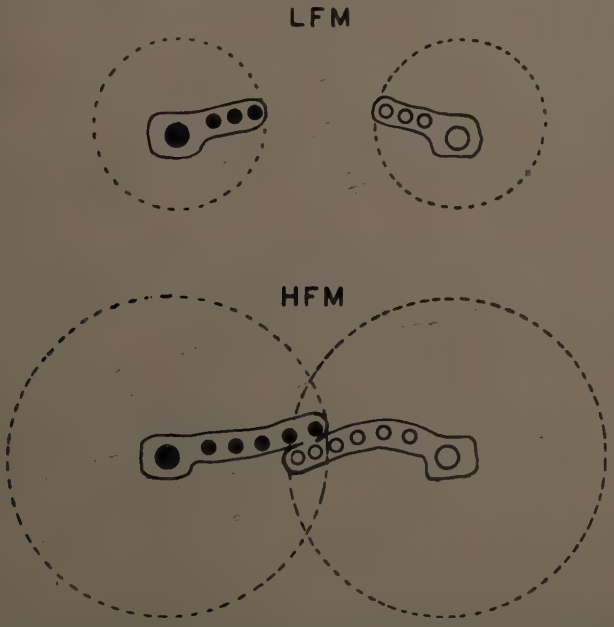

FIGURE 7. One hypothetical basis for the difference in frequency of heterokaryon formation between HFM (high-frequency mating) and LFM (low-frequency mating) strains. The residual germination of the LFM strains on selective (minimal) agar is not sufficient to permit direct contact (*upper drawing*), while extensive residual growth of germinated HFM spores separated by the same distance allows for hyphal contact (*lower drawing*). This hypothesis was not confirmed experimentally (see text).

by the HFM property, since no successful crosses were achieved. It was observed for *S. fradiae*, however, that in intraspecific crosses involving an HFM parent the heterokaryotic frequency was 5 to 10 times higher than in crosses with an LFM parent (Braendle *et al.*, 1959).

Synkaryosis

While heterokaryosis results in the emergence of a new phenotype, which reflects the combination of dominant parental characters within a single mycelium, this condition is unstable even on selective media, since the uninuclear conidia are always of parental genotype. Sermonti and Spada-Sermonti (1955) observed, however, that stable recombinant conidia were obtained in crosses between auxotrophic mutants of *S. coelicolor*. Since then, a considerable amount of data has been accumulated describing, in addition to genic recombination in various strains of *S. coelicolor* (Braendle and Szybalski, 1957; Hopwood, 1957; Bradley, 1957, 1958), this type of interaction in other *Streptomyces*, including *S. fradiae* (Braendle and Szybalski, 1957), *S. rimosus* (Alikhanian and Mindlin, 1957) and *S. griseoflavus* (Saito, 1958).* One of us (D.H.B.) also has observed recently the production of prototrophic recombinants from one pair of *S. griseus* mutants (TABLE 5), although only heterokaryotic interactions were detectable between all other marked strains of this species.

The experimental methods leading to the production of recombinants included the following steps: (1) propagation of a large mass of heterokaryotic or mixed mycelium under selective conditions for a prolonged period of time with accompanying sporulation; (2) plating of the conidia on various selective media which did not support the growth of the parental types; and (3) isolation of putative recombinant colonies, purification, and characterization of the one or more strains derived from each colony. In the case of *S. fradiae* and *S. griseoflavus*, balanced heterokaryons grew reasonably well on minimal medium supplemented with various nonparental combinations of amino acids (10 μg./ml.). This first experimental step, however, was modified for *S. coelicolor*, which does not form nutritionally balanced heterokaryons. The mixture of parental strains of this species was cultured on nutrient agar for a period of 8 days. The details of the experimental procedure have been described previously (Braendle, 1957; Braendle and Szybalski, 1957).

Our own studies on synkaryosis included, in addition to the experiments with (1) *S. fradiae*, recombination analysis of (2) streptomycin-resistant strains of *S. coelicolor*, derived from nutritional mutants supplied by G. Sermonti and from (3) *S. coelicolor* N.L. 9021 (TABLE 1), (4) mutants of *S. coelicolor* obtained from D. A. Hopwood, (5) mutants of *S. griseoflavus* obtained from H. Saito, (6) mutants of *S. rimosus* supplied by S. I. Alikhanian, and finally (7) a few mutants of *S. griseus*. Exemplary results of crosses performed by one of us (D.H.B.) between strains of *S. fradiae*, *S. coelicolor*, and *S. griseoflavus* are shown in TABLES 6 to 8. For the other crosses, either insufficient data are available as yet (TABLE 5), or accurate analysis of the data was prevented by technical difficulties, as for example, the high reversion rates for the his$^-$ and

* Elsewhere in this monograph, K. F. Gregory presents evidence for genetic recombination in *Streptomyces scabies*.

TABLE 6

RECOMBINANTS RECOVERED FROM CROSSES BETWEEN MUTANTS OF *S. FRADIAE*

(Crosses Reciprocal for Streptomycin Resistance)

	Parental strains		Number and type of recombinant colonies recovered from minimal medium supplemented with:															
			met + his				met + arg				isl + his					isl + arg			−−
	6F4-1	6F5-16	2	30	16	1	47	2	2	13	1*	1*	3*	29*	1*	27	21	1*	48
Colony type																			
his	+	−	+	+	−	−	+	+	+	−	+−	−−	++	+−	+−	+	+	++	+
arg	+	−	+	+	+	+	+	−	+	+	++	++	++	++	+−	+	−	+−	++
met	−	+	+	−	+	−	+	+	+	+	++	+−	−+	−+	−+	+	+	++	++
isl	−	+	+	+	+	+	+	+	+	+	++	++	++	++	++	+	+	++	+
SM	s	r	r	s	r	r	r	r	r	r	r r	r r	s r	s r	s r	r	r	r r	r
Class†	p	p	R	i	a	A	R	h	R	a	R a	a A	i R	i a	i a	R	h	R h	R
			met + his				met + arg				isl + his			isl + arg					−−
	6F4-10	6F5-13	13	27	1*	1*	10	17	22	1*	48	1	1*	6	2	19*	5*	10*	50	3
Colony type																				
his	+	−	+	+	−+	++	+	+	+	++	+	−	+−	+	+	++	+−	++	+	+−++
arg	+	−	+	+	−+	++	+	+	−	+−	+	+	++	+	−	+−	+−	++	+	+−+−
met	−	+	+	−	−+	−+	+	−	+	++	+	+	++	+	+	−+	−+	−+	+	++−−
isl	−	+	+	+	++	++	+	+	+	++	+	+	−+	+	+	++	++	++	+	++−+
SM	r	s	s	r		s s	s	r	s	s s	s	s	r s	s	s	r s	r s	r s	s	
Class†	p	p	R	i	A R	A R	R	i	h	R h	R	a	m a	R	h	i h	i p	iR	R	R p p A

* Colonies containing a mixture of two or more types of conidia.

† Key: R, prototrophic recombinant; A, auxotrophic recombinant with the arrangement of markers not attributable to the single prototrophic reversion; a, h, i, m, auxotrophic recombinants, or one-step revertants at arg, his, isl/leu, and met loci respectively; p, parental combination of markers. For symbols and abbreviations, see TABLE 1.

TABLE 7

RECOMBINANTS RECOVERED FROM CROSSES BETWEEN MUTANTS OF *S. COELICOLOR*

(Crosses Reciprocal for Streptomycin Resistance)

	Parental strains		Number and type of recombinant colonies recovered from minimal medium supplemented with:																					
			met + glu				his + glu					his + pro			met + pro						---			
	14D1-2	14D2	8	7	3	2*	12	1	5	1*	2*	8	1	6	5	2	4	7	1	1*	12	6	1*	1*
Colony type																								
met	−	+	+	+	+	++	+	+	+	++	++	+	+	+	+	+	+	+	+	+−	+	+	++	++
his	−	+	+	+	+	++	+	+	+	++	++	+	+	+	+	+	+	+	+	++	+	+	+−	++
pro	+	−	+	+	+	++	+	+	+	+−	++	+	+	−	+	+	+	−	−	+−	+	+	++	++
glu	+	−	+	+	+	++	+	+	+	++	++	+	+	+	+	+	+	+	+	++	+	+	++	++
pigm	+	−	+	−	±‡	+−	+	−	±‡	++	+−	+	−	−	+	−	±‡	+	±†	++	+	±‡	+−	+−
SM	r	s	r	r	r	r r	r	r	r	r r	r r	r	r	r	r	r	r	r	r	r r	r	r	r s	r r
Class†	p	p	R	R	R	R R	R	R	R	R A	R R	R	R	A	R	R	R	A	A	R A	R	R	R A	R R

			met + glu			his + glu						his + pro						met + pro							---			
	14D1	14D2-1	18	1	1	7	1	4	2	1*	1*	4	1	4	8	1	2	3	4	1	6	2*	3*	1*	11	2	6	1*
Colony type																												
met	−	+	+	+	−	+	+	+	+	+−	++	+	+	+	+	+	+	+	+	−	−	−−	−−	−+	+	+	+	++
his	−	+	+	+	+	+	+	−	−	++	−+	+	+	+	−	−	−	+	+	+	+	+−	++	++	+	+	+	++
pro	+	−	+	+	+	+	+	+	+	−+	−+	+	+	+	+	+	−	+	−	+	−	−+	−+	++	+	+	+	++
glu	+	−	+	+	+	+	+	+	+	++	++	+	+	+	+	+	+	+	+	+	+	++	++	++	+	+	+	++
pigm	+	−	+	±‡	+	+	−	+	−	+−	++	+	−	±†	+	−	+	+	+	+	−	−+	−+	++	+	−	±‡	+−
SM	s	r	s	s	s	s	s	s	s	r s	s s	s	s	s	s	s	s	s	s	s	s	r s	r r	r s	s	s	s	s s
Class†	p	p	R	R	h	R	R	m	A	A A	A R	R	R	R	m	A	A	R	A	h	A	A p	A A	A R	R	R	R	R R

* See TABLE 6.

† See TABLE 6.

‡ Pigmentless colony, producing pigmented sectors.

TABLE 8

RECOMBINANTS RECOVERED FROM CROSS BETWEEN TWO MUTANTS OF *S. GRISEOFLAVUS*

	Parental strains		Number and type of recombinant colonies recovered from minimal medium supplemented with:*																									
			met + try							met + gly							ade + gly									–			
	1G1	1G2	1	4	1†	1†	1†	1†	1†	3	21	1†	1†	1†	1†	1†	6	1	2	1†	5†	1†	1†	1†	2†	25	1	1†	1†
Colony type																													
ade	−	+	+	+	++	+−	++	−+	+++	+	+	++	++	+−	−−	−++	+	−	+	++	−+	−−	−++	−−	−+	+	+	++	+−
met	−	+	+	−	+−	+−	+−	−−	+−+	+	−	+−	+−	−−	+−	−+−	+	+	+	++	−+	+−	−−+	+−	−+	+	+	+−	−−
gly	+	−	+	+	++	++	++	++	−++	+	+	++	++	++	−+	+−+	+	+	+	++	++	++	+++	++	+−	+	+	++	++
try	+	−	+	+	++	−+	−+	++	−++	+	+	++	−+	++	−+	+−+	+	+	−	+−	+−	−+	+−+	++	+−	+	−	++	++
Class‡	p	p	R	d	R d	g p	g d	p d	p d R	R	d	R d	g d	d p	A p	p p d	R	m	g	R g	p g	A p	p d g	m p	p p	R	g	R d	d p

* Confluent syntrophic growth on minimal agar supplemented with tryptophan and glycine prevented the use of this selective medium for isolating recombinants.

† See TABLE 6.

‡ See TABLE 6; d, g, m: auxotrophic recombinants, or revertants at ade, gly, met loci, respectively.

met⁻ loci of Hopwood's *S. coelicolor* strains and the lack of sporulation in one of Alikhanian's *S. rimosus* mutants.

Our experimental results have the following features in common with the findings of most other workers in the field: (1) the prevalence of prototrophic recombinants, even on media selective for different recombinant types; (2) the common occurrence of colonies containing approximately equal numbers of two or more recombinant or prototrophic strains; (3) the detection of only a few of all the possible types of nonparental recombinants; and (4) the stability of purified prototrophic and auxotrophic recombinants upon subculture. The only observations at variance with these points were cited by Hopwood (1957), who reported the isolation of all possible recombinants in theoretically predictable ratios in his strains of *S. coelicolor*, and by Bradley (1958), who observed segregation of rare parental-type conidia from presumably purified stocks of prototrophic recombinants in still another strain of *S. coelicolor*.

Before dwelling further on the experimental findings, it may be useful to discuss briefly the analogies to the genetic mechanisms in other micro- and macroorganisms. All types of direct interaction between two genomes can be classified according to one of two criteria: (1) completeness, that is (*a*) fusion of two complete genomes, (*b*) fusion of two partial genomes, or one complete with one partial genome; and (2) stability, that is (*c*) formation of a stable zygote, which is either diploid (if mechanism *a* operated) or carried some chromosomes in a hemizygous or haploid condition (if mechanism *b* operated), or (*d*) segregation of the two genomes with or without preservation of their identity. The modes of interaction between complete genomes are presented in a schematic, abbreviated form in FIGURE 8. Each genome in the haploid state is represented by two chromosomes of different length. From the formation of an unstable diploid phase, haploidy may be re-established through one of two pathways: (1) meiosis, an orderly, specialized mechanism through which concomitant crossing-over and reduction lead to the production of all reciprocal combinations of markers in predictable ratios; and (2) the parasexual cycle, first described by Pontecorvo (1953), in which the chromosomal exchanges take place during the diploid phase (mitotic crossing-over) independently from the haphazard reduction process (haploidization). The hypothetical mechanism of haploidization in the parasexual cycle involves the loss of chromosomes during nondisjunctive or other faulty mitotic divisions, with resulting progression through several unstable aneuploid states to the stable haploid phase (Pontecorvo and Käfer, 1958). The haploid-diploid-haploid cycle results only in exchanges of whole chromosomes, while the additional rare intervention of mitotic crossing-over during the diploid phase also produces intrachromosomal rearrangements. To this schematic picture should be added the mechanism involving partial participation of one genome with its complete or incomplete integration, and the borderline case of a nonduplicating piece of the genome, as in the phenomenon of abortive transduction.

Which of these mechanisms operate in *Streptomyces* synkaryosis? To answer this question on the basis of the presently available data is difficult for at least two reasons: the apparent discrepancy in the results obtained for various strains or species, and the possibility that two or more mechanisms may operate simultaneously. The scarcity of experimental data due to tech-

nical difficulties associated with work on the streptomycetes further delays the solution to this question. The mechanisms proposed here are based as much on positive evidence as on the exclusion of other possibilities. We shall discuss synkaryotic interaction from the aspects outlined earlier in this section:

(1) The paucity of markers and the existence of technical problems have not permitted the design of experiments that would demonstrate conclusively whether complete or incomplete genomes participate in synkaryosis. How-

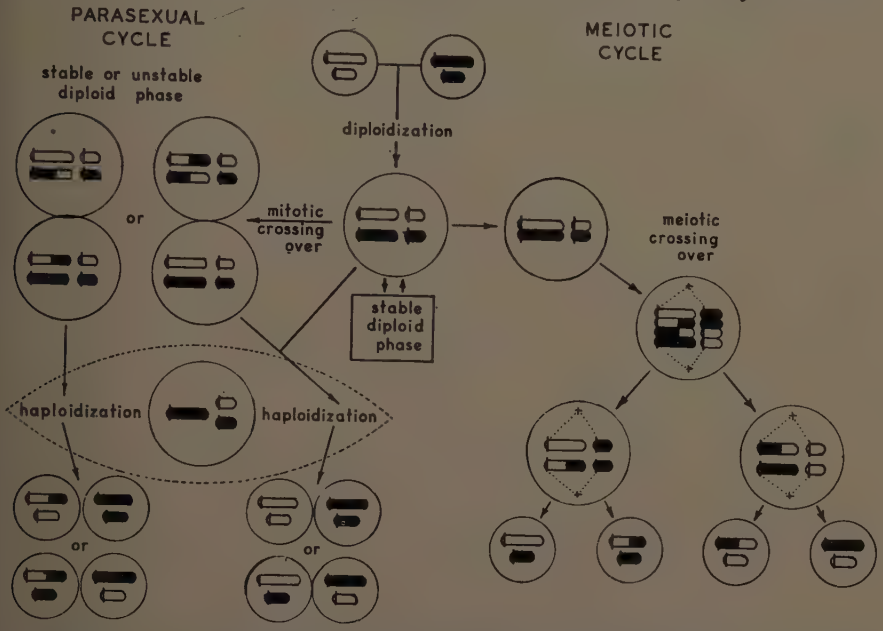

FIGURE 8. Abbreviated, diagrammatic representation of the parasexual and meiotic cycles In the parasexual cycle, the diploid nucleus can undergo mitotic crossing-over, which may result in establishment of experimentally detectable homozygosity for the distal markers on one chromosomal arm (*upper far-left pair of cells*). Through the independent process of haploidization (haphazard loss of chromosomes, symbolized by the cell with 3 chromosomes), different types of haploid cells can be derived. Haploids derived directly from diploids are either identical to one of the haploid parents, or show exchange of whole chromosomes (recombination between unlinked markers; *lower center-left pair*). Haploids derived from the diploid products of mitotic crossing-over exhibit intrachromosomal exchanges (recombination between linked markers; *lower left pairs*).

In meiotic reduction division, the processes of crossing-over and reduction proceed in a well-coordinated manner. In the absence of special selective influences, each allelic pair of markers should segregate according to a 1:1 ratio (*lower right*).

ever, multiple exchanges of markers point to the participation of complete genomes, or at least of their greater part. Thus, mechanisms analogous to transduction or transformation may be excluded for the time being.

(2) Stability of the diploid phase arising from the fusion of two haploid nuclei could not be demonstrated in the majority of the experiments. In spite of the prevalence of prototrophic recombinants in crosses between multiple auxotrophic parents, three kinds of evidence contraindicated the formation of stable heterozygous diploids analogous to those of the filamentous fungi (Pontecorvo, 1953):

(*a*) The prototrophic recombinants were stable and no parental segregants were ever isolated, either for *S. coelicolor* or *S. fradiae*, even by a special screening system designed to recover putative segregants as rare as 1 per 10^{10} conidia (Braendle and Szybalski, 1957). In this procedure, crosses were made between streptomycin-sensitive and streptomycin-resistant, doubly auxotrophic mutants, and conidia of the streptomycin-sensitive prototrophic recombinants were plated on streptomycin-containing nutrient agar. Diploids heterozygous for auxotrophy and streptomycin resistance and, therefore, phenotypically sensitive to this antibiotic, would be expected to segregate out the streptomycin-resistant parental auxotroph. However, no such segregants appeared on the streptomycin-containing nutrient agar, while the few streptomycin-resistant colonies isolated under these conditions were all prototrophic and thus of mutational origin.

(*b*) Crosses reciprocal for streptomycin resistance yielded either predominantly resistant or sensitive prototrophic recombinants (TABLES 6 and 7), while presumptive diploids heterozygous for resistance should have been uniformly streptomycin-sensitive. The experimental findings indicate clearly that the prototrophic recombinants were not diploid, at least for the streptomycin-resistance locus. All the streptomycin-resistance markers employed (*S. fradiae*) were tested in heterokaryotic combinations and found to be uniformly recessive to streptomycin sensitivity.

(*c*) Auxotrophic recombinants were back-crossed to parental or other recombinant strains and were found to behave in the manner predicted for haploid strains, as discussed by Hopwood elsewhere in this monograph. This finding excludes the possibility that these recombinants could have been the stable diploid products of mitotic crossing-over.

Thus, stable diploidy of the type described for *Aspergillus* and *Penicillium* seems to be absent in *Streptomyces*. The only possible exception is the observation of Bradley (1958) which, however, requires more critical quantitative evaluation. On the other hand, the existence of a very transient diploid phase may be indicated by the many mixed recombinant colonies encountered during the search for synkaryonts (TABLES 6 to 8). Other interpretations, however, could be advanced for this mixed growth obtained from a filtered conidial inoculum, including either inadvertent plating of hyphal fragments containing adjacent recombinant and parental nuclei, or secondary heterokaryosis and, possibly, even recombinations between synkaryonts and the excess of parental conidia under the experimental conditions employed for isolation of recombinants.

(3) Since stable diploidy has not been detected, reduction division must follow closely after synkaryosis. A classic meiotic division seems improbable, because of the failure to recover all the predicted reciprocal products, as reported by most authors reporting in this monograph. However, Hopwood's results, also, presented in this monograph, are compatible with the meiotic mechanism. On the other hand, haploidization alone, as postulated for fungi by Pontecorvo and Käfer (1958), could also account for the presence of haploids that recombined for unlinked markers only. To invoke the parasexual mechanism for explaining the recombination between linked markers, it would be

necessary to postulate a very efficient mechanism of mitotic crossing-over operating during the very short diploid phase.

(4) Finally, let us consider the possibility of the existence of stable hemizygous recombinants, with only a segment of chromosome in the diploid heterozygous condition. The data presented by the Sermontis and by Alikhanian elsewhere in this monograph favor this possibility, but critical evidence is still needed. If such were the case, distinction between prezygotic and postzygotic elimination of the remainder of the genome would require further technical refinements in the experimental methods applied to *Streptomyces*.

All these considerations seem to point to a closer resemblance of *Streptomyces*, in mode of sexual reproduction, to Eubacteriales, as exemplified by *Escherichia coli* K-12, than to the filamentous fungi, although the coenocytic mycelium and resulting heterokaryotic interaction seem to indicate a superficial relationship to the latter group.

Purposeful Breeding of Streptomyces

Since a sexual cycle in *Streptomyces* was not known until recently, selection of spontaneous and induced mutants has been employed extensively as the sole method of improving the economically important strains, mainly from the point of view of antibiotic yields. Most of the strains currently used for the production of a given antibiotic originated from the same partially improved stock, but evolved through different pathways in the course of their development by various industrial laboratories. The desirable properties of several of these independently developed high producers could be combined in a single strain and the unsuitable traits eliminated by making use of the sexual cycle in *Streptomyces*. If high-producing strains of diverse origin were not available, crossing of present day high yielders with the original parent strain, followed by repeated backcrossing to the parental high producer, might result in the isolation of improved strains due to replacement of deleterious characters accumulated during the long history of strain development. Another promising application of synkaryosis is the improvement of actinophage-resistant mutants by repeated backcrosses to the parental high yielder from which they were derived and isolation of phage-resistant recombinants.

For the purposeful breeding of *Streptomyces*, mutants with genetic markers not affecting antibiotic productivity must be isolated and crossed on selective media. Large-scale assay of a multitude of recombinants and further crosses between the best of them should produce the desired results. The methods outlined would be admittedly laborious, but not more so than presently employed purely mutational work. The first and only program of this type for *Streptomyces*, to our knowledge, is outlined by S. I. Alikhanian elsewhere in this monograph. The pioneering work of G. Sermonti with the mold *Penicillium* is an example of a similar approach with another organism. Because of the magnitude of the effort required, only large industrial research laboratories, with their vast facilities for quantitative fermentative evaluation of a multitude of strains, have the potential to undertake successfully such a genetic breeding program. When applied in conjunction with mutational and chemical analytical studies, this type of program could lead not only to drastic improvement

in the yields and cultural characteristics of industrial strains, but also to the production of chemically modified and superior fermentation products, including antibiotics. The possibilities inherent in intelligent application of genetic methods to the purposeful breeding of industrial streptomycetes appear to be boundless.

Summary

To isolate a sufficient number of genetically marked strains of *Streptomyces*, new techniques were developed or conventional methods, including ultraviolet irradiation, the filtration enrichment technique, and multipin replication, were adopted. More than 200 single and multiple mutants carrying nutritional, resistance, and morphologic markers were isolated. Heterokaryosis was observed only between mutants derived from a common parental strain of *S. fradiae*, *S. griseus*, *S. griseoflavus*, *S. parvus*, *S. venezuelae*, *S. albus*, or *S. spheroides* and, more rarely, between mutants derived from different strains of the same species (*S. griseus*). In *S. fradiae*, a heterokaryotic compatibility system was characterized. When two freshly isolated auxotrophic mutants were mated on minimal medium, 1 to 10 heterokaryotic colonies were formed from 10^8 conidia of each parent. On the other hand, when secondary parental strains isolated from the conidia produced by these heterokaryotic colonies were mated, the frequency of heterokaryon formation increased by a factor of 1000 or more. The property of high-frequency mating could be regarded as a genic character selected for in a single step, and reasonably stable upon serial subculture of the strain.

Heterokaryons were utilized in the study of various properties of *Streptomyces*. Individual conidial chains derived from a heterokaryon were shown to be composed of a mixture of both parental types of spores, which bears on the mechanism of conidia formation. In heterokaryons, high neomycin productivity behaved as a dominant character over low productivity, while streptomycin or catenulin resistance was recessive to streptomycin sensitivity. Nutritional deficiencies were recessive in most cases. Occasionally with some species and routinely with others, however, nutritionally unbalanced heterokaryons were observed. Formation of heterokaryons between two strains could be considered as a criterion for close taxonomic relationship.

Within heterokaryons, synkaryotic interaction was observed with the production of stable genetic recombinants. Synkaryosis was studied in *S. fradiae*, *S. griseoflavus*, and in various strains of *S. coelicolor* and *S. griseus*. The recombination data indicate that the diploid phase is of very transient nature with subsequent early haploidization and exchange of genetic characters. The long period intervening between actual haploidization and scoring of the recombinant colonies made quantitative evaluation of the results difficult, because of the strong selective pressures acting during multiplication, sporulation, and germination of recombinants.

Synkaryotic recombination is potentially useful for the purposeful breeding of *Streptomyces* to obtain higher yields and to modify the chemical structure of economically important metabolites, including antibiotics.

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Discussion of the Paper

K. F. Gregory (*Ontario Agricultural College, Guelph, Canada*): The discovery that it is possible to reisolate from a heterokaryon of *Streptomyces fradiae* secondary parental strains exhibiting a greatly increased frequency of heterokaryon formation is particularly exciting. If this phenomenon proves to be a general rule for species of *Streptomyces* it should provide a simple technique that will greatly facilitate genetic studies with these organisms. It appears to be possible that some pairs of mutant strains, apparently unable to

heterokaryose may, by preliminary heterokaryotic association with other strains, be induced to do so.

Some of my experiences during investigations on *S. scabies*, the causative agent of potato scab, relate directly to the techniques and conclusions presented by W. Szybalski and D. H. Braendle, and I should like to describe these briefly. For some time, I have utilized the filtration-enrichment technique as an aid to the selection of auxotrophic mutants of this species. Germination of some conidia of this species in liquid minimal medium, especially following irradiation, was delayed so long that mycelium from the first germinated spores had made extensive growth before the remaining spores germinated. In order to avoid the feeding of auxotrophic spores by this extensive prototrophic mycelium and yet allow most of the prototrophic spores to germinate, it was found necessary to pass the culture through sterile filter paper on each of three successive days. The repeated manipulations involved, however, frequently resulted in bacterial contamination, a difficulty that was overcome partly by starting with a streptomycin-resistant mutant and incorporating streptomycin into the media employed.

Approximately 50 per cent of the auxotrophic mutants isolated from one strain of *S. scabies* required a reduced form of sulfur (for example, cysteine, thiosulfate) for growth. This type of mutant was encountered only once in a second strain of *S. scabies*. These mutants, called parathiotrophic by Pontecorvo *et al.*,[1] have been reported to comprise a large proportion of the auxotrophic mutations in *Aspergillus nidulans* but, to my knowledge, have not been encountered previously in large numbers among the streptomycetes or true bacteria.

The strain of *S. scabies* selected for heterokaryon and recombination studies had been shown previously by cytological studies to undergo frequent hyphal fusion. Structures believed to be septa were commonly formed in old hyphae, and occasionally in young ones. Application of the lysozym technique for protoplast formation, as described by R. J. Douglas of my laboratory, to this culture caused the production of protoplastlike bodies confirming the theory that some of the protoplasm, at least, occurred in the mycelium in discrete pockets. Several spherical chromatinic bodies, following the acid-Giemsa stain technique, were found within each cell, as delineated by the septa, and these cells thus were considered to be multinucleate. Although all the cytological evidence indicated, therefore, that heterokaryon formation should be possible in this culture, several combinations of diauxotrophic strains derived from it failed to produce prototrophic heterokaryons. Attempts to produce heterokaryons with mutants derived from another strain of *S. scabies* and between *S. scabies* and *S. griseus* were unsuccessful. One pair of strains (A26-32, requiring nicotinic acid and biotin, and A26-33, requiring arginine and methionine), however, readily formed slow-growing prototrophic colonies on minimal agar at the rate of about $30/10^6$ spores plated. These colonies were considered to be heterokaryons since the majority of spores formed by them possessed the nutritional requirements of one or the other of the two parents, and growth on new minimal medium was obtained only by mycelial transfer. Whereas the formation of heterokaryons between two strains appears to be a good criterion for a close relationship or identity, as concluded by Szybalski and Braendle,

it is clear that the failure of such heterokaryons to form does not necessarily indicate nonidentity or distant relationship.

An appreciable number of spores formed by the heterokaryon required only nicotinic acid or only biotin. In addition, one of every 500 to 1000 viable spores required both methionine and biotin for growth; one requirement derived from each of the parents. These characteristics were stable and transmitted through the spores. This combination of auxotrophic characteristics is not believed to be caused by nutritionally unbalanced or anomalous auxotrophic heterokaryons, since spores of this strain are uninucleate and haploid according to cytological (acid-Giemsa stain), radiological (strictly logarithmic rate of killing with X rays), and mutational evidence. Spores with the reciprocal requirements (arginine + nicotinic acid) were not found. Although many details of the genetic interaction between these auxotrophs of *S. scabies* remain obscure, it appears that synkaryosis and genetic recombination occur in this species.

Reference

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GENETICS OF *STREPTOMYCES COELICOLOR*

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Streptomyces coelicolor is the first species in the genus *Streptomyces* in which gene recombination has been observed[1] and the one that has been most widely studied.[2-7] A tentative picture of the gene recombination process in this species, considering all the data reported so far, will be presented in this paper.

Strain

S. coelicolor Reiner-Müller is classified in the family *Streptomycetaceae* of the order *Actynomycetales*.[18] The colonies on a corn-steep medium at neutral *p*H consist of a colorless primary mycelium and a thick layer of whitish aerial mycelium carrying conidia in chains. The medium becomes intensely colored by a blue soluble pigment formed by the organism, which turns red and becomes insoluble at acid *p*H.

Cytological observations with a thionine stain of the conidia of our isolate revealed a single stained body per conidium.[4] In a different strain (*S. coelicolor* 9023) rare conidia carrying rodlike, dumbell-shaped or double bodies have been observed in addition to a large majority of conidia containing single stained bodies (Robinow's staining).[8] Radiokinetic experiments with our strain[9] indicate haploid nuclei in the conidia, and the mutation rates after ultraviolet treatment[2, 6] also suggest a single haploid nucleus per conidium.

Mutations

The media used, the cultural conditions, the details of the techniques, and the types of mutants obtained in *S. coelicolor* will not be reported in this note since they may be found in the papers quoted below.

Nutritional mutants have been selected among colonies surviving ultraviolet treatment by total isolation[2] or after replica plating.[4, 6] Enrichment by the filtration technique also has been used.[4]

Mutants resistant to antibiotics have been studied, particularly by Braendle and Szybalski,[4] and mutants resistant to virulent phages by Bradley.[6] Temperate phages have not been detected in this species.[10]

Strains decomposing agar, as well as strains differing in sugar requirements and morphological characteristics have been described by Stanier,[11] who remarked their peculiar instability. Erickson[12] observed variations comprising loss of pigmentation, loss of aerial mycelium and agar liquefaction; he also noted an inherent tendency for a given mutation to occur more frequently in one clone than in another. Color mutants, lacking the blue pigment characteristic of the species, have been described.[2, 6] Most of these show a reddish or pink primary mycelium.

The markers most widely used in gene recombination studies were nutritional markers, first, because they are most likely of genic nature, and second, because balanced heterokaryons, heterozygous diploids, or haploid recombinants

can be selected easily in fungi or in bacteria when the parental strains are complementary auxotrophs.

Heterokaryosis

Heterokaryosis in *S. coelicolor* has been looked for as a step preliminary to nuclear fusion and gene recombination. Using nutritionally marked strains, we attempted a synthesis of balanced heterokaryons.[2] Possible occurrence of heterokaryons has been explored in mixed cultures of complementary auxotrophic strains. In the mixed culture a remarkable syntrophic overgrowth takes place. This is particularly evident when two colonies, belonging to different auxotrophic strains, grow side by side on an agar medium. A tuft of exuberant growth appears in the zone of contact between the adjacent colonies, which contrasts with them in height and richness of spore formation.[2]

Transfer of such tufts on minimal medium gives rise to stunted colonies with an irregular edge and greatly delayed spore formation. Spores from such colonies, as well as spores harvested directly from the syntrophic tufts, give rise only to parental colonies. No growth has been observed from several tens of thousands of hyphae from the above-mentioned colonies after plating on minimal medium.[2]

The syntrophic tufts have been considered as nutritionally unbalanced heterokaryons by Braendle and Szybalski,[4] but no conclusive evidence of the occurrence of different nuclei in the same hyphal fragment has been presented so far. Bradley[5, 6] considers that heterokaryosis occurs infrequently in the mixed cultures, and is perpetuable through conidial transfers. The extremely low rate of segregation he obtains from the supposed heterokaryons, however, points to a close interrelationship between the component genomes in such clones, which can hardly be described as heterokaryosis. Assuming a random segregation of the two types of nucleus of a heterokaryon upon conidiation, the frequency of homokaryotic conidia should be equal to $2^{-(n-1)}$, where n is the number of nuclei per conidium. Since Bradley[6] reports a frequency less than 0.1 per cent ($\simeq 2^{-10}$) of homokaryotic conidia from the heterokaryotic clones, this would require an average of about 10 nuclei per conidium.

Although gene recombination in *S. coelicolor* has been reviewed repeatedly under the title heterokaryosis,[13, 14] this stage has not yet been conclusively demonstrated in this species.

Recombinants from Conidia of Mixed Cultures

Rare recombinant phenotypes can be detected by plating millions of conidia produced by a mixed culture of two genetically marked strains onto selective agar media. When the parental strains are double auxotrophic mutants, both prototrophic and auxotrophic recombinants can be selected by plating conidia of the mixed culture on partially supplemented media.[2-4]

When the medium of the mixed culture is not selective in favor of the recombinant phenotypes, the rate of recombinant colonies per plated conidia of the mixed culture[7] ranges between 10^{-4} and 10^{-5}, but the nonselective action of the medium can never be assured safely.

An attempt to map several genes on the basis of the frequency of the detected recombinant phenotypes has been made by Hopwood.[3] He observed that

complementary recombinant phenotypes appeared with similar frequencies. His data have not yet been published in full.

Experiments dealing with the detection of recombinants among conidia from mixed cultures are characterized by a very poor reproducibility. The types of detectable recombinants and their relative frequencies vary widely from experiment to experiment, and the causes of such variability as yet have not been discovered. Braendle and Szybalski[4] assume that strong selective forces intervene in the period between the occurrence of recombination and the scoring of the recombinant colonies.

Segregation from Recombinant Phenotypes

In our first work[2] recombinant phenotypes were tested on the assumption that they were pure and stable clones. This was, in fact, the case with some of them.[2] Bradley[5] observed segregation of the parental types from some prototrophic recombinant clones, as well as occurrence of exceptional recombinant auxotrophic phenotypes. On the basis of these observations he used the term heterokaryon to designate the prototrophic recombinant phenotypes. Braendle and Szybalski[4] have described, among the colonies obtained after plating of conidia from a mixed culture on selective media, "colonies containing a mixture of approximately equal numbers of two or more types of conidia." The observations mentioned point to the occurrence in some of the single conidia (or units) initiating the recombinant clones of both alleles of at least some of the markers involved and to a "segregation" following the plating of the conidia of the mixed culture.

This possibility has been explored further in the following experiments.[7] Fourteen recombinant colonies from cross 14 me hi s^r (methionineless, histidineless, streptomycin-resistant) × 23 pr glu pigm (prolineless, glutamateless, no pigment produced) have been considered. They were selected on minimal medium supplemented with proline and methionine, or with proline and histidine. Spores from each of the recombinant colonies have been plated on a complete medium, and small samples of the arising subclones have been tested for nutritional requirements. Seven of the 14 recombinant colonies examined contained 2 or more types of conidia and, of these, 4 contained 2 or more types of recombinant conidia (TABLE 1). The parental types obtained from the conidia of the recombinant colonies cannot be considered safely as segregant types, since the recombinant colonies were growing on media containing sufficient parental conidia, whose requirements were, however, not satisfied in the medium. Due to the high frequency of the parental conidia recovered in some of the colonies and to the complete lack of observable growth in the selective medium, the parental types obtained from the recombinant colonies should be considered as probable segregants.

To detect possible rare subclones whose requirements were not satisfied in the plating medium (MM + proline + methionine), the spores of some recombinant colonies were streaked on a second selective medium containing the supplements omitted in the first selective medium (histidine and glutamate). From the streaks spores were then plated on complete medium and the arising colonies tested for color and for nutritional requirements.[7] In this way, up to four different phenotypes have been detected in subcultures of some recom-

binant colonies. Some of them carried nutritional requirements not satisfied in the first selective medium, and others showed phenotypes selected against in both selective media; among the latter some were parental types (TABLE 2, clone 75).

In a third experiment, the segregant types from recombinant colonies were screened in such a way as to provide a rough estimation of their frequency. Recombinant colonies (cross: 14 × 23, on MM + methionine + proline) were transferred on slants, and conidia from the slants were plated on complete and on selective media, to recover the segregant types. The results are shown in TABLE 3. Rare subclones have been found in all the clones in which they were looked for. Prototrophic subclones from conidia of essentially prolineless

TABLE 1

DIFFERENT PHENOTYPES RECOVERED FROM CONIDIA OF SINGLE RECOMBINANT COLONIES (Cross: 23(s^r) me hi × 14 pr glu (pigm)*)

Plating medium† (MM +)	Recombinant colony (Code No.)	Recovered phenotypes		Plating medium† (MM +)	Recombinant colony (Code No.)	Recovered phenotypes	
		No.	Symbol‡			No.	Symbol‡
Methionine and proline	2, 3, 5	16	+	Histidine and proline	17	16	pr
Methionine and proline	1, 9	16	me	Histidine and proline	7	6	pr
						10	+
Methionine and proline	10	16	pr	Histidine and proline	14	14	pr
						2	me hi
Methionine and proline	8	12	+	Histidine and proline	8	6	pr
		4	me hi			8	+
Methionine and proline	6	6	pr			2	me hi
		1	+	Histidine and proline	15	10	pr
		9	me hi			1	+
Methionine and proline	11	9	+			5	me hi
		2	me				
		5	pr				

* The characters between parentheses were not considered in this experiment.

† Medium on which conidia of the mixed culture have been plated to select recombinant colonies; MM, minimal medium.

‡ Symbols of mutant characters represent the phenotype; + means prototrophic.

colonies were detected at a rate of about 10^{-4}, and new auxotrophic subclones at rates of about 10^{-5}.

Recombinant colonies from mixtures of strains of different colors give rise to either homogeneous or heterogeneous offspring, in regard to the color markers. A heterogeneous clone, derived from cross 23 × 14, has been studied during several subtransfers.[7] Its conidia gave rise to either blue pigment-producing, or pink pigmentless prototrophic colonies. Upon transfer the blue colonies bred true, while the pink ones gave a number of blue among a majority of pink derivatives. This behavior was attributed[7] to heterozygosis of the pink clones and to the dominance of the absence of pigment (pigm) over the wild condition.

Bradley[6] observed the rare segregation of parental types from some recombinant auxotrophic phenotypes, which at a still lower rate also produced prototrophic derivatives. He considers these recombinants as anomalous hetero-

karyons, in analogy to those observed by Bradley and Lederberg[15] in *S. griseus*. The so-called anomalous heterokaryons in *S. coelicolor* are, however, extremely stable, as compared with those described in *S. griseus*, and again the use of the term heterokaryon does not describe the situation properly.

The occurrence of several auxotroph segregants that exhibit a nutritional requirement not recognized in either parent has also been reported by Bradley.[6] This observation is of extreme interest, pointing to an enhanced rate of mutation occurring during the process leading to gene recombination.

TABLE 2
DIFFERENT PHENOTYPES RECOVERED FROM SINGLE RECOMBINANT CLONES AFTER RESTREAKING ON SELECTIVE MEDIA
(Cross: 23 (s^r) me hi × 14 pr glu pigm*)

First plating medium† (MM +)	Recombinant clone (Code No.)	Second selective media‡ (MM +)	Recovered phenotypes	
			No.	Symbol§
Methionine and proline	35	methionine and proline	45	pr
		histidine and glutamate	16	+
			5	glu pigm
			13	pr
			4	pr glu
Methionine and proline	44	methionine and proline	43	pr
			2	+
		histidine and glutamate	43	+
Methionine and proline	61	methionine and proline	44	pr
		histidine and glutamate	10	hi glu
Methionine and proline	75	methionine and proline	44	pr
		histidine and glutamate	2	+
			4	pr
			11	me hi
			9	hi glu

* See first note, TABLE 1.
† See second note, TABLE 1.
‡ Only colonies producing growth on the two second plating media and practically no growth on MM have been further analyzed.
§ See third note, TABLE 1.

A remarkable parental probable segregant from a prototrophic recombinant (cross: 23 s^r me hi × 203 ur arg) has been observed by one of us (G. S.). Its colonies had the phenotype of parent 23 (s^r me hi), but some of them exhibited overgrowing sectors and papillae that turned out to be histidine independent (s^r me). Parental colonies of strain 23, in similar conditions, did not show the same instability.

Discussion

The recombination of the genetic material of two different strains of *S. coelicolor* has been shown by the occurrence of recombinant phenotypes that

appear to breed true on unselective media. The processes that give rise to the recombinant phenotypes involve some type of pairing of the nuclei originally carried by the parental strains. The degree of integration, however, of the parental genomes is still in question. According to Bradley (elsewhere in this monograph), a heterokaryotic or dikaryotic condition initially is set up which eventually leads to a true nuclear fusion. The occurrence of a heterokaryotic stage was inferred from the observation that prototrophic recombinant strains

TABLE 3
FREQUENT AND RARE SUBCLONES FROM SINGLE RECOMBINANT CLONES*
(Cross: 23 (s^r) me hi × 14 pr glu pigm†)

Recombinant clone (Code No.)	Frequent subclones			Rare subclones§			
	No.	Phenotypes‡	Frequency per 10^2 conidia	Selective medium (MM +)	No.	Phenotypes‡	Frequency per 10^6 conidia
24	30	pr	100	methionine	9	+	158
				histidine & glutamate	40		
					1	hi glu	6.5
38	30	pr	100	methionine	55	+	216
				histidine & glutamate	35		
					2	hi glu	9.6
4	30	pr	100	methionine	40	+	257
				histidine & glutamate	59		
21	30	pr	100	methionine	23	+	77
					4	me	13.3
35	227	+	87	not tested			
	33	me	13				
3, 20, 22, 27	30	+	100	not tested			

* Isolated on MM + methionine and proline.
† See first note, TABLE 1.
‡ See third note, TABLE 1.
§ Detected after plating dense suspension of conidia of the corresponding clone on the selective media.

gave rise to auxotrophic segregants of which the vast majority were of the parental type.[6] This observed preferential segregation could be due to an incomplete integration of the parental genomes (conjugate division of nuclei without actual fusion). It could also be due, however, to biased scoring of the segregant colonies or to linkage relationships between markers in the heterozygous nuclei. More elaborate quantitative evidence is required in order to arrive at a firm conclusion.

It cannot be decided at this time whether the process of nuclear fusion involves two entire genomes or only parts of them (merozygosis[16]). Some tentative idea of the nature of the zygotes can be derived from the results of segrega-

tion in recombinant colonies. For this purpose two assumptions must be made: (1) that the recombinant clones, selected after plating conidia of mixed cultures, arise from uninucleate cells; and (2) that all of the markers recovered from the subclones were present in the nucleus of the original cell.

The first assumption has been questioned by Bradley,[6] who feels that conidia might be multinucleate, although cytological observations and induced mutation rates indicate the contrary. It must be borne in mind, however, that both the cytological and the mutational data refer to conidia of parental cultures sampled at random, and yet the recombinant clones could be derived from an infrequent and peculiar unit. However, it would appear unlikely that single conidia contain sufficient nuclei to permit the many possible nuclear ratios supposed by Bradley,[6] or to give rise to the various recombinant subclones that have been detected by us.[7] Recombinant heterogeneous colonies might be derived also from occasional clusters of conidia or from fragments of mycelium present among the conidia plated on the selective medium. However, this possibility would appear unlikely, since the filtered conidial suspension shows clusters of conidia or fragments of mycelium so infrequently that they could not explain the observed high frequency of the heterogeneous recombinant colonies.

The second assumption is also questionable. Two groups of subclones detectable from the recombinant colonies must be distinguished: those that appear at a high rate and those that appear infrequently. When the two alleles of a gene are detected at a high frequency among the conidia of the same colony, it may be concluded that both were present in the original nucleus (or cell). The rare segregants however, admit other possible interpretations. One is the occurrence in the recombinant nucleus of either forward or backward mutations at an exceptionally high rate. The recovery by Bradley[6] of new mutations among segregant phenotypes is in agreement with this suggestion. Another interpretation is the possible occurrence of delayed and infrequent integration into recombinant nuclei of genetic information present in the original nucleus (or cell) in some inactive state. This could explain most of the rare segregants and revertants observed by Bradley,[6] the rare recombinant subclones detected by us,[7] the occasional segregation from pigmentless colonies of blue pigmented colonies,[7] and the appearance of revertant sectors and papillae from a segregant colony as reported in this paper. All of these interpretations are possible at the present stage of research with *S. coelicolor*. Far more data still must be gathered before a final conclusion may be reached.

The data at the present time suggest that in *S. coelicolor* rare nuclear fusions occur in the mycelium of a mixed culture. Such nuclear fusions may lead to either incomplete or complete zygotes. These nuclei undergo segregation processes, which are sometimes complete before the conidiation of the mixed culture, giving rise to pure recombinant colonies after plating of the conidia. In some instances the segregation process continues or perhaps even begins after the formation of the conidia. Colonies derived from such partially or completely heterozygous conidia form a mixture of different subclones. This tentative picture of recombination in *S. coelicolor* is in complete agreement with that proposed by Saito[17] in *S. griseoflavus*, and with the observations by Braendle and Szybalski[4] in *S. fradiae*. These species, however, differ from *S. coeli-*

color by the possession of a balanced heterokaryotic state. To complete the picture of the recombinational cycle in *S. coelicolor*, the probable occurrence of some kind of genetic instability connected with the recombination process must be envisaged.

Acknowledgment

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CYTOGENETIC STUDIES ON *STREPTOMYCES GRISEOFLAVUS*

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Genetic and cytological investigations of the streptomycetes have been greatly stimulated in recent years by the importance of these organisms in the industrial production of antibiotics. Knowledge of the genetics of microorganisms progressed rapidly during the past decade, and many new avenues are now open that permit a study on the genetics of streptomycetes. We undertook a study of the genetics of *Streptomyces griseoflavus*, strain 142, late in 1954. This strain was first isolated from soil in Japan and its ability to produce vitamin B^{12} was recognized. However, as conidiation of this strain on appropriate media was rapid and abundant, this strain was considered promising for genetic studies. While the details of these investigations have been published as a number of individual papers, we shall summarize here the whole of our work in this field.

In attempts to induce biochemical mutants we found that the isolation of such mutants was difficult when using the classic technique of ultraviolet irradiation. One explanation for this difficulty was the possibility that the strain we were using was not haploid; we therefore undertook a cytogenetic study especially on the ploidy of the conidial nuclei. Our investigations also suggest the possible occurrence of genetic recombination in heterokaryons. Although our data are not yet sufficient to infer a precise mechanism for genetic recombination in the *Streptomyces*, we feel it is of interest and trust that it may at least serve as a basis for discussion.

Occurrence of Genetic Recombination[1, 2]

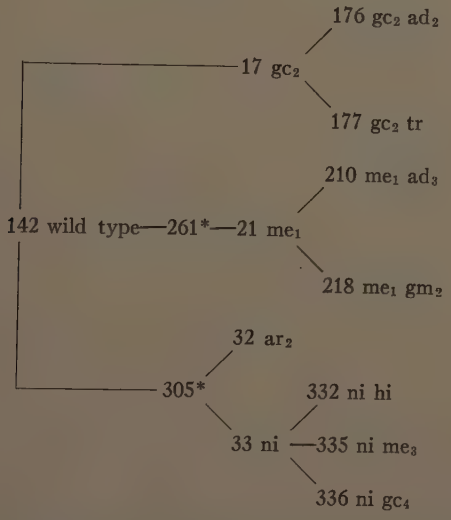
Auxotrophic mutants, induced by ultraviolet irradiation of the wild-type strain of *S. griseoflavus* 142 were used throughout the studies. FIGURE 1 gives the cell lineage of mutant strains.

Experiments designed to test for genetic recombination were carried out in a system similar to that used by Sermonti and Spada-Sermonti.[3] Two auxotrophic mutants were inoculated on the same agar slant of limiting medium, which was composed of Czapek's medium plus 0.03 per cent yeast extract and 0.1 per cent peptone. Heterokaryotic growth was observed after 2 to 4 days' incubation at 30° C. only in the area where the two mutants mingled. Cross-feeding was almost negligible in every case. The conidia produced on the heterokaryotic hyphae were harvested after 8 to 12 days' total incubation and suspended in sterile saline after thorough washing. By means of mechanical vibration and filtration through a glass filter, more than 80 per cent of the conidia were separated.

The conidial suspensions were plated on minimal (Czapek's) and supplemented media. The supplements added did not permit the growth of either parental strain, but would allow the growth of recombinants. For example, when 177 gc_2 tr and 210 me_1 ad_3 had been crossed, the diluted conidial suspension was spread on five types of media, namely the minimal medium and medium supplemented with any of the following combinations: glycine and

methionine, glycine and adenine, tryptophan and methionine, or tryptophan and adenine.

After 72 hours' incubation at 30° C., plates were scanned for recombinant colonies. Such colonies were recorded by marking, and the plates were then incubated for several more days to await conidial maturation. The nutritional requirements of recombinant colonies were determined by the replica-plating technique. For example, in the above-mentioned cross, the colonies on medium, supplemented with glycine and methionine, were tested for their growth responses on the minimal medium and on the media supplemented with glycine and/or methionine. The replica plates were incubated for 2 days before inspec-



* Strains 261 and 305 exhibit slow growth on minimal medium and nonspecific responses to compounds such as isoleucine and cysteine.

FIGURE 1. Lineage of mutant strains. Key to nutritional requirements: ar, arginine; gm, glutamic acid; gc, glycine; hi, histidine; me, methionine; tr, tryptophan; ad, adenine; and ni, nicotinic acid.

tion. The types and frequencies of recombinants determined for each cross are shown in TABLE 1. Some of the recombinants picked up at random, as well as all recombinants involving two marker characters, were isolated and verified in subculture.

Occasionally the recombinant colonies were seen to produce more than one type of conidia; therefore, we examined the homogeneity of conidia as follows. A large number of conidia were removed from a recombinant colony with the aid of a platinum inoculating needle, suspended in saline, and inoculated onto complete agar (solidified nutrient broth containing glucose). The nutritional requirements of the resulting secondary colonies were evaluated by the replica-plating technique employing minimal medium and 5 types of supplemented media that would detect each type of recombinant from the cross. For example, in the case of 176 gc_2 ad_2 × 335 ni me_3, 14 types of recombinants are

possible; these can be determined on 5 media supplemented with GAN, GAM, GNA, ANM, and GANM (G, glycine; A, adenine; N, nicotinic acid; and M, methionine).

As shown in TABLE 2, 22 among 73 recombinant colonies studied were found to show segregation on conidiation. All except 2 of these heterogeneous colonies produced conidia of 1 recombinant type together with either or both of the parental types. The occurrence of such conidia might be due to the formation of heterokaryons between the recombinants and parental mycelial growth. However, the 2 exceptional colonies, indicated by asterisks in TABLE

TABLE 1

FREQUENCIES OF SELECTED RECOMBINANTS AMONG CONIDIA PRODUCED ON MIXED CULTURES

Cross (parents)	Experiment number	Number of conidia			Cross (parents)	Experiment number	Number of conidia		
		Total tested	Recombinant*				Total tested	Recombinant*	
177 gc_2 tr × 210 me_1 ad_3	I	1×10^5	+	120	176 gc_2 ad_2 × 335 ni me_3	I	1×10^5	+	4
			tr	210				ni	146
			me	40				ad	184
								me	22
	II	1×10^8	+	4				ad ni	4
			me	38				ad me	1
			tr	1					
177 gc_2 tr × 332 ni hi	I	1×10^6	+	0	177 gc_2 tr × 335 ni me_3	I	1×10^6	+	330
			hi	298				ni	135
			tr	34				me	28
								tr	2
								tr me	1
	II	5×10^6	+	0		II	7×10^7	+	430
			hi	823				ni	177
								me	56
			tr	17				tr	23
210 me_1 ad_3 × 332 ni hi	I	3×10^7	+	0	210 me_1 ad_3 × 336 ni gc_4	I	1×10^8		0
			me	66					
			hi	15		II	1×10^8		0
			me ni	3					
			ad hi	1		III	1×10^6	+	0
			me hi	1				me	3

* The symbol + designates a prototrophic type.

2, segregated 2 recombinant types and a parental type. The most reasonable explanation for these latter findings is the coexistence of more than 1 gene set in the conidium.

The mutants used in these studies were fairly stable, as adjudged in terms of spontaneous reversions. By analyzing conidia harvested from cultures on media supplemented with various concentrations of nutrients, the reversion frequencies of me_1 and hi were found to be 1×10^{-7} or less per conidium, and no reversions were detected with other markers. Bradley suggested in his recent paper the occurrence of enhanced mutation and selection in heterokaryons of *S. coelicolor*.[4] In our studies, certain types of single auxotrophs predominantly were recovered, as shown in TABLE 1. These data might be

explained by postulating frequent reversions at gc_2, ad_3, ni, and me_3 and the selection of revertant nuclei in heterokaryons. However, the frequent occurrence of prototrophs may also, and perhaps more probably, be due to the exchange of genetic material between two unlike nuclei. The recovery of double

TABLE 2
SEGREGATION IN RECOMBINANT COLONIES WITH CONIDIATION

Cross	Recombinant colony tested			Type† (number) of conidia found on the colony
	Medium (supplement)	Type†	Number	
177 gc_2 tr × 210 me_1 ad_3	minimal	+	11	+ (all)
176 gc_2 ad_2 × 335 ni me_3	minimal	+	9	+ (all)
	adenine nicotinic acid	ad	4	ad (all)
	adenine nicotinic acid	ni	4	ni (all)
	adenine methionine	ad	3	ad (all)
	adenine methionine	ad	1	ad(7), ni me(6)
	adenine methionine	ad	1*	ad(20), ni(8), ni me(10)
	adenine methionine	me	1	me (all)
	adenine methionine	me	1	me(8), gc ad(7)
	adenine methionine	me	1*	me(10), ni(2), gc ad(10)
177 gc_2 tr × 335 ni me_3	trypophan methionine	+	1	+ (all)
	trypophan methionine	+	3	+(83), ni me(22)
	trypophan methionine	tr	1	tr(50), ni me (22)
	trypophan methionine	me	1	me (all)
	trypophan methionine	me	4	me(96), ni me(75)
	trypophan methionine	me	2	me(57), ni me(21), gc tr(36)
210 me_1 ad_3 × 332 ni hi	methionine histidine	hi	6	hi (all)
	methionine histidine	me	1	me (all)
	methionine histidine	me	2	me(49), ni hi(25)
	methionine histidine	me	1	me(120), me ad(45), ni hi(35)
	methionine nicotinic acid	me	1	me (all)
	methionine nicotinic acid	me	1	me(21), ni hi(15)
	methionine nicotinic acid	me ni	3	me ni (all)
177 gc_2 tr × 332 ni hi	glycine histidine	hi	4	hi (all)
	glycine histidine	hi	1	hi(182), ni hi(28)
	tryptophan histidine	hi	2	hi (all)
	tryptophan nicotinic acid	tr	3	tr(77), ni hi(65)

* These colonies segregate two recombinant types.
† The symbol + designates a prototrophic type.

auxotrophs showing nonparental combination of nutritional requirements, in fact, gives strong support to the belief that genetic recombination occurs in *S. griseoflavus*.

Possible Mechanisms of Genetic Recombination

Genetic interaction without plasmogamy, such as desoxyribonucleic acid (DNA) transformation, may be improbable in *S. griseoflavus*. One of us (H. S., at the Department of Microbiology, Yale University, New Haven, Conn.)

has been engaged in a number of trials of DNA transformation without success. The experiments involved the treatment of germinating conidia and partially disintegrated hyphae with lysozym. Bacteriophage-mediated transduction might be another possibility. However, no phage system has been detected in the present strain.

On the other hand, plasmogamy in *S. griseoflavus* was evidenced by the formation of heterokaryons. Upon 4 to 5 days' incubation of two auxotrophic mutants plated together on minimal medium, heterokaryotic colonies producing only the parental type of conidia were formed. Mycelial transfer to successive subcultures gave rise to prototrophic growth. Some of the long hyphal fragments that were about 20 μ in length, when isolated by micromanipulation, appeared to be heterokaryotic although the shorter fragments were either homokaryotic or inviable. In many cases, conidiation of these heterokaryotic colonies on minimal medium was slow and poor. Upon prolonged incubation, dense growth attributed to recombination appeared on areas of the radial sparse growth of heterokaryotic mycelia.

Under these conditions it is probable that genetic recombination occurs in the heterokaryon following the karyogamy, which might be incomplete. Similar interpretations were proposed by Braendle and Szybalski[5] and by Hopwood.[6] Taking into consideration the segregation data of recombinant colonies, we tentatively postulate the formation of hemizygotes or merozygotes, which are occasionally formed in heterokaryotic hyphae. Most of the zygotes may segregate into haploid nuclei before conidiation.

In our cytological studies on the life cycle of *S. griseoflavus*,[7] we have observed that the primary mycelium gives rise to three diverse and distinctive structures, namely, "nests," "swollen bodies," and "clubs." It is an attractive speculation that some of these organs may play a role in genetic interaction. Klieneberger-Nobel[8] has proposed that nuclear material of the primary mycelium undergoes zygosis, giving rise to diploid nuclei that subsequently migrate into the secondary mycelium. If this is the case, it is also possible that diploid conidia, when heterozygous, segregate into recombinants. We shall discuss this problem in the following sections.

X-ray Irradiation Studies on Conidia[9]

Conidia of *S. griseoflavus* 142 were harvested from a slant culture on Krainsky's medium containing 1 per cent polypeptone and 0.25 per cent yeast extract, and were suspended in sterile saline. More than 90 per cent of conidia were single after repeated filtration through a glass filter. Ten ml. of diluted conidial suspension containing 10^5 conidia/ml. were put into a sterile beaker, 1 in. in diameter, and subjected to X-ray irradiation. We used a Matsuda KXC-18 type X-ray projector at 200 Kvp and 20 mAmp. The X ray, was filtered through a 1.0 mm. aluminum plate and the intensity was 1000 r/min. at the distance of 18 cm. where the conidial suspension was placed. At intervals, aliquots of the suspension were diluted and plated in triplicate on complete medium. The plates were incubated for 4 to 5 days at 30° C. before counting.

The survival curves of wild-type conidia were sigmoidal, and the hit number of the curves was about 2.3. The same curves were obtained using conidia of different ages (FIGURE 2). Two mutant substrains, which were derived from

the wild-type strain 142 after ultraviolet irradiation, were examined in a similar way. The conidia of these strains exhibited somewhat different sensitivity to the X-ray irradiation, but again gave survival curves of the two-hit type (FIGURE 3). This latter characteristic is seen to be hereditarily stable.

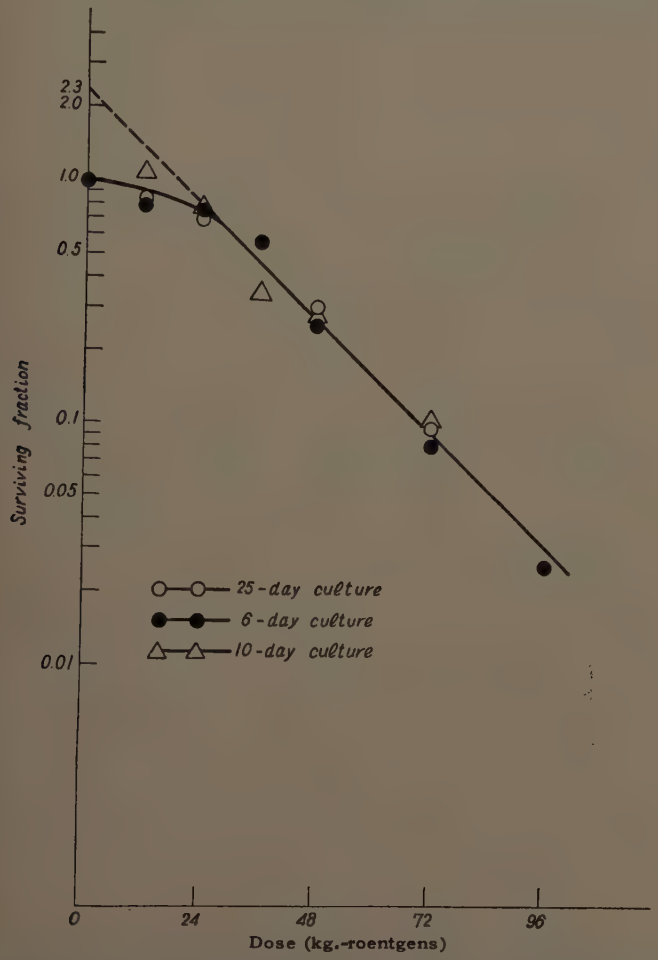


FIGURE 2. Survival after X irradiation of *S. griseoflavus* 142. Reproduced by permission from the *Journal of General and Applied Microbiology*.

In contrast to these situations, conidia of *S. griseus* No. 1, *S. coelicolor* (by courtesy of G. Sermonti), and *S. kitasatoensis* exhibited exponential curves. When a suspension of hyphal fragments, harvested either from an agar slant or from a submerged culture of *S. kitasatoensis*, was subjected to irradiation, the survival curve was of a multihit type (FIGURE 4). Since this strain conidiated scarcely at all on the modified Krainsky's medium or in the young submerged culture, the suspension after filtration contained only hyphal fragments, which were 10 to 50 μ in length. The average number of nuclear

granules in individual fragments, stained by Robinow's nuclear staining technique, was approximately 10; this value corresponded well to the hit number. We have interpreted these findings as evidence for the presence of two sets of genetic units in a conidium of *S. griseoflavus*. Elsewhere in this monograph, Dr. Bradley indicated that, according to his experience, the X-ray irradiation of

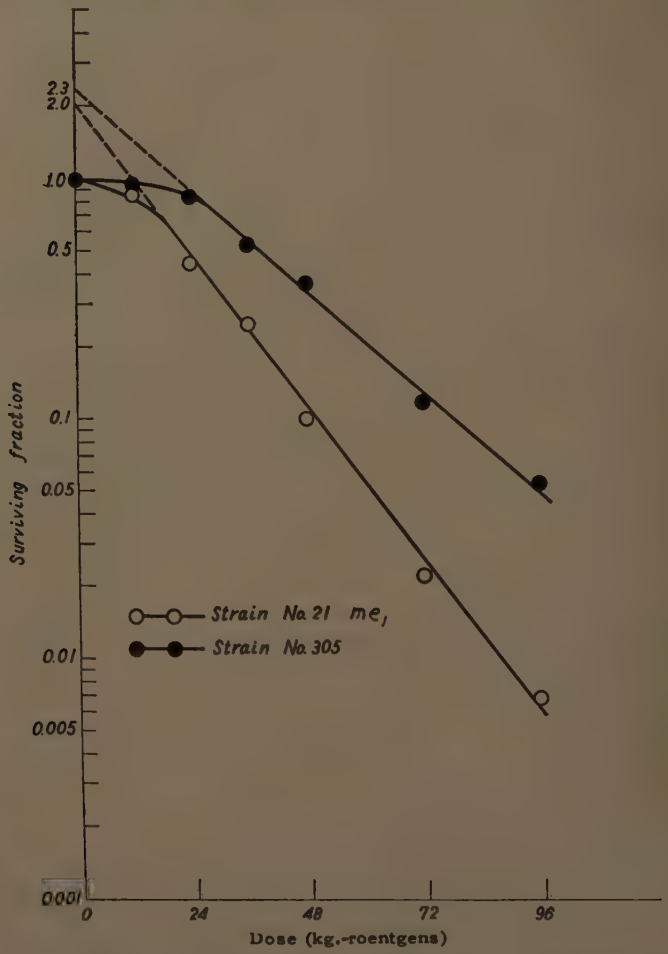


FIGURE 3. Survival after X irradiation of mutant strains of *S. griseoflavus*. Reproduced by permission from the *Journal of General and Applied Microbiology*.

S. coelicolor gave a sigmoidal survival curve with tailing. This is also true in our experiment; when plotting the survivals only at low doses of irradiation, the hit number of the curve is more than one (see FIGURE 4-II).

Mutation and Segregation in Conidia of S. griseoflavus

As mentioned in the introduction, we encountered difficulty in detecting auxotrophic mutants among clones established by plating immediately follow-

ing irradiation with ultraviolet light. The following procedure, however, did permit the recovery of auxotrophic strains.[10] Washed conidia, at a density of approximately 10^8/ml., were suspended in 10 ml. of sterile saline or minimal medium. They were then subjected to irradiation with ultraviolet light in sufficient dosage to produce 99 to 99.9 per cent kill. The treated conidia were then incubated on a complete agar medium for 7 to 10 days at 30° C. Conidia

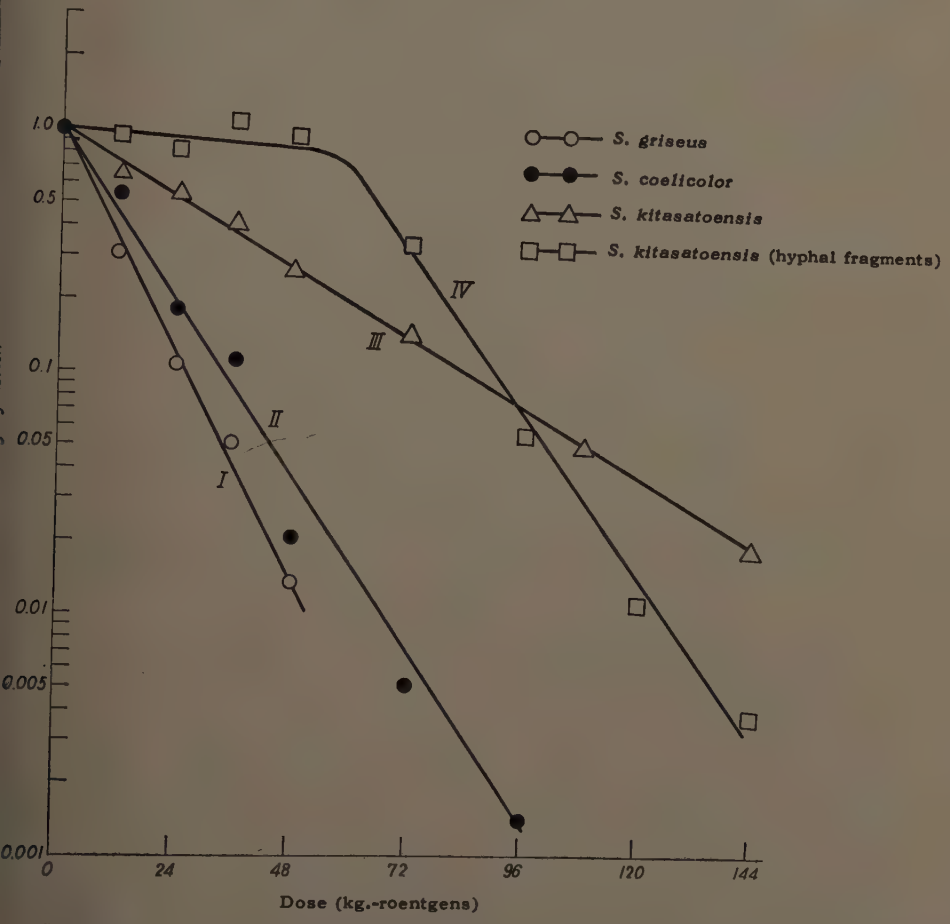


FIGURE 4. Survival after X irradiation of *S. griseus*, *S. coelicolor*, and *S. kitasatoensis*. Reproduced by permission from the *Journal of General and Applied Microbiology*.

produced on this culture were then washed and incubated in minimal liquid medium. During the incubation period hyphal growth was filtered off every day. After 5 to 6 days the remaining conidia were plated on complete medium and their growth factor requirements were examined by replica plating.

As shown in TABLE 3, the frequency of detected auxotrophs was enhanced by the additional growth cycle. These data may be explained as the result of the delayed phenotypic appearance. It is known that the genes controlling nutritional traits are recessive in other organisms. Therefore, if the conidia

of *S. griseoflavus* have diploid nuclei, induced mutation in one of two homologous chromosomes should not express itself phenotypically before segregation. Thus, these data again suggest the diploid nature of conidia.

This conclusion is supported further by the observation that revertant conidia arising from auxotrophs may be heterozygous for the character reverted.[11] This can be shown in the following experiment. Two mutant strains, 218 me_1 gm_2 and 32 ar_2, were used for this study. The gm_2 and ar_2 genes were known to revert spontaneously to prototrophs at appreciable frequencies. For the study of induced mutation, about 10^9 washed conidia were suspended in 10 ml. sterile saline and subjected to ultraviolet irradiation. The surviving conidia, approximately $10,^7$ were plated on minimal medium or supplemented medium that would allow only the growth of revertants. Revertant colonies appeared in 2 days' incubation at 30° C., but were incubated

TABLE 3
ENHANCEMENT OF MUTANT RECOVERY BY PREINCUBATION

Parent strain	Procedures for detecting mutants*	No. of colonies examined	No. of mutants detected	Frequency of mutant detection
No. 142 wild	UV.....repl.	approx 50,000	1†	2.0×10^{-5}
No. 261	UV.....filt....repl.	approx. 2,500	2	8.0×10^{-4}
No. 305	UV.....filt....repl.	approx. 2,000	3	1.5×10^{-3}
No. 305	UV.....filt....repl.	approx. 1,000	1	1.0×10^{-3}
No. 142 wild	UV...incub....filt....repl.	430	4‡	9.3×10^{-3}
No. 21 me_1	UV...incub....filt....repl.	400	5‡	1.3×10^{-2}
No. 33 ni	UV...incub....filt....repl.	700	4‡	5.7×10^{-3}
No. 17 gc_2	UV...incub....filt....repl.	1,500	4‡	2.7×10^{-3}

* Key: UV, UV-irradiation; incub., preincubation on complete medium; filt., filtration process; repl., replica-plating method.
† This mutant exhibits slow growth on minimal medium.
‡ Numbers represent different types of mutants.

for an additional 3 to 6 days to permit maturation. A number of conidia were removed from the center of the single revertant colonies by means of a platinum needle. These were streaked on complete agar and incubated for 5 to 8 days to mature the growth of new (or secondary) colonies. The nutritional requirements of these secondary colonies were determined by the replica-plating technique. When at least 100 secondary colonies were of one type, the revertant colony was considered homogeneous.

As shown in TABLE 4, 20 among 29 revertant colonies tested were heterogeneous; that is, they had produced conidia of both reverted and auxotrophic types. The frequencies of genuine revertants among the tested secondary colonies are shown in TABLE 5. These genuine revertants were no longer heterogeneous, as far as 33 colonies tested were concerned.

The possibility exists that parental-type conidia remained viable on the minimal agar, although no apparent growth was observed. A revertant conidium, homozygous per se, could form a heterokaryon with these parental conidia. The resulting heterogeneous colony would produce two types of

conidia, the wild and the mutant. Reconstruction experiments were undertaken to test this hypothesis. Five to 10 revertant (or wild-type) conidia, which had been made homozygous through single-colony isolation, were plated

TABLE 4
HETEROGENEITY OF REVERTANT COLONIES DERIVED FROM AUXOTROPHIC MUTANTS OF *S. GRISEOFLAVUS*

Strain	Reverted locus	UV treatment (min.)	Survivals (percentages)	Reversion frequency among survivals	Number of revertant colonies			
					Tested	Heterogeneous	Homogeneous	
							Reverted type	Auxotrophic type
218 me_1 gm_2	gm_2	0	100	4.1×10^{-7}	7	7	0	3
		1	25	2.6×10^{-6}	13	9	2	2
32 ar_2	ar_2	0	100	1.0×10^{-6}	6	4	2	0

TABLE 5
RATIO OF REVERTED CONIDIA DETECTED IN HETEROGENEOUS REVERTANT COLONIES DERIVED FROM 218 me_1 gm_2

UV treatment (min.)	Ratio of revertants to total number of conidia tested
0	3:53, 8:26, 6:18, 24:42, 20:30, 35:46, 38:40
1	80:100, 12:32, 188:200, 7:20, 10:15, 10:23, 21:24, 200:203, 26:40

TABLE 6
RECONSTRUCTION TEST

Strain	Background parent conidia (No. conidia per plate)	Number of colonies			
		Tested	Heterogeneous	Homogeneous	
				Independent type	Auxotrophic type
218-1 me_1*	218 me_1 gm_2 (10^7)	14	0	13	1
218-2 me_1*		15	0	15	0
142 wild		17	0	17	0
32-1*	32 ar_2 (10^6)	10	0	10	0
142 wild		15	0	15	0

* The strains are revertants which have been isolated from either 218 me_1 gm_2 or 32 ar_2.

with 10^7 conidia of the parent-mutant type on a supplemented medium identical to that used in the preceding experiments. The colonies developing on these plates were examined as described above. No heterogeneous colonies were found, as may be seen in TABLE 6. These results strengthen the possibility that the revertant colony developed from a conidium containing two types of genomes: revertant and wild type.

Cytological Studies on Conidia

We have made a cytological study of the life cycle of *S. griseoflavus* using Robinow's staining technique.[7] Modified Krainsky's medium was used for slide-glass cultures similar to the method used by Klieneberger-Nobel.[8] The growth was fixed for 3 min. in the vapor of 2 per cent osmic acid, hydrolyzed in 1 *N* HCl at 60° C. for 10 min., and finally stained with Giemsa's stain (1:10 dilution) for 10 min.

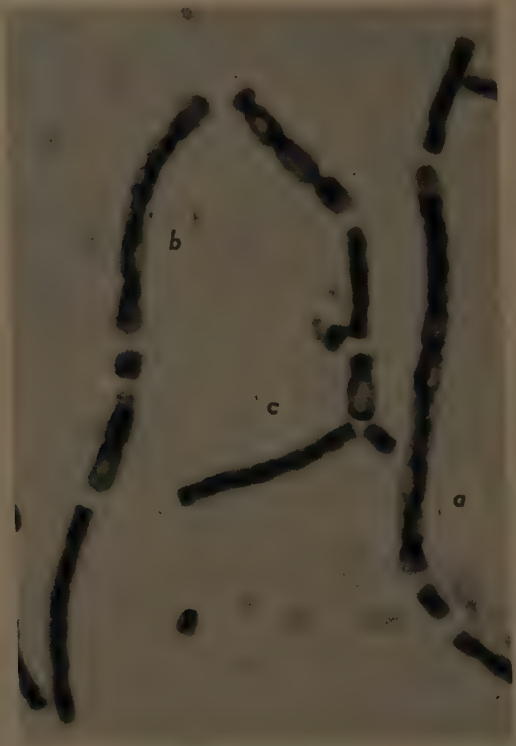


FIGURE 5. Conidiation by segmentation: (*a*) segmentation of cytoplasm; (*b*) chromatinic dumbbell formation; (*c*) maturation. Reproduced by permission from *Cytologia*.

It seemed to us that there were two different processes of conidiation. One was initiated by the segmentation of secondary mycelium that contained deeply staining cylindrical structures. The other type was a fragmentation developing directly from primary mycelium (FIGURES 5 and 6). In both processes, the nuclear structures in immature conidia were frequently rodlike or dumbbell-shaped. These structures were found even after fragmentation of hypha and formation of individual conidiospore cases. The majority of mature conidia, however, contained single spherical nuclei (FIGURES 7 and 8). Therefore, it seemed to us that the dumbbell form of the staining bodies may represent two sets of genetic material that later condense to form the spherical nucleus of a single conidium.

Discussion on the Status of Nuclei in Conidia

Recently Kinoshita and Itagaki reported the results of cytological studies of conidia of a number of species of *Streptomyces*.[12] They found that the majority of mature conidia were uninucleate (FIGURE 9), but that a number of strains produced binucleate conidia, as well as some containing dumbbell- or rod-shaped nuclei (FIGURES 10 and 11, and TABLE 7). It seems to us that these figures reflect diverse evolutional steps in *Streptomyces*. The majority of the streptomycetes produce uninucleate conidia, and the nuclei may be haploid.

FIGURE 6. Fragmentation (*middle*) in conidiation; twisted rods and dumbbell-shaped chromatinic substances are observed. Reproduced by permission from *Cytologia*.

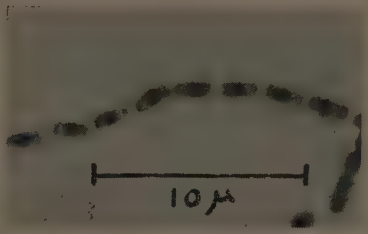


FIGURE 7. Mature conidia containing single spherical nuclei. Reproduced by permission from *Cytologia*.

However, some strains may have diploid or bipartite nuclei which exist in apparently uninucleate conidia. Binucleate or dikaryotic conidia also may be produced by some strains. The species producing only uninucleate conidia may be the most primitive. Genetic recombination may occur only in the relatively higher streptomycetes, such as *S. coelicolor*, *S. fradiae* 3535, and *S. griseoflavus* 142. Comparative studies of these various strains from both the genetic and cytological points of view would provide interesting information.

In *S. griseoflavus*, it is probable that the nucleus in a conidium consists of two sets of genetic units in spite of its appearance as an uninucleate conidium. We have assumed that bipartite nuclei are formed by the nuclear duplication

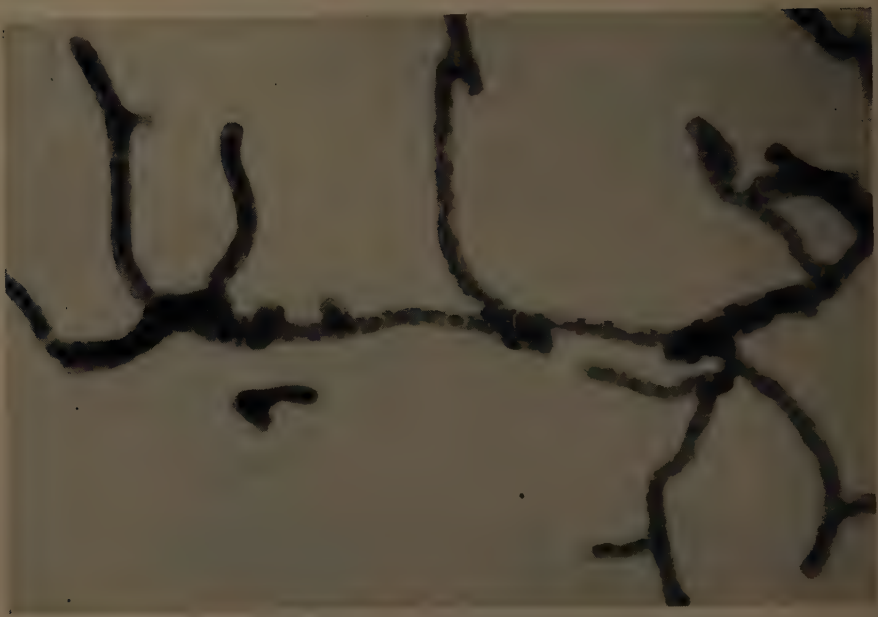


FIGURE 8. Mature conidia of *S. griseoflavus*. Adapted from work reported by Kinoshita and Itagaki.[12]

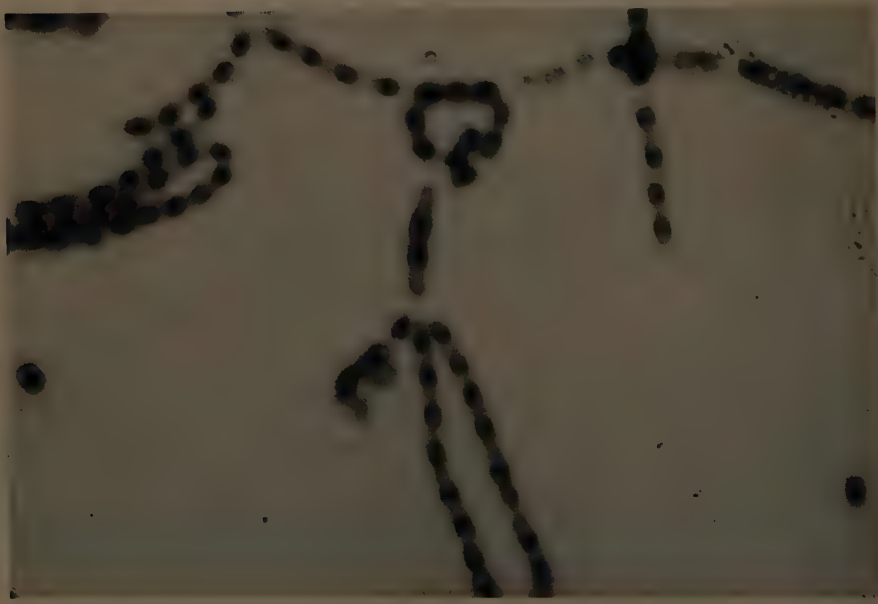


FIGURE 9. Conidia of *S. aureus* ATCC 3309. Adapted from work reported by Kinoshita and Itagaki.[12]

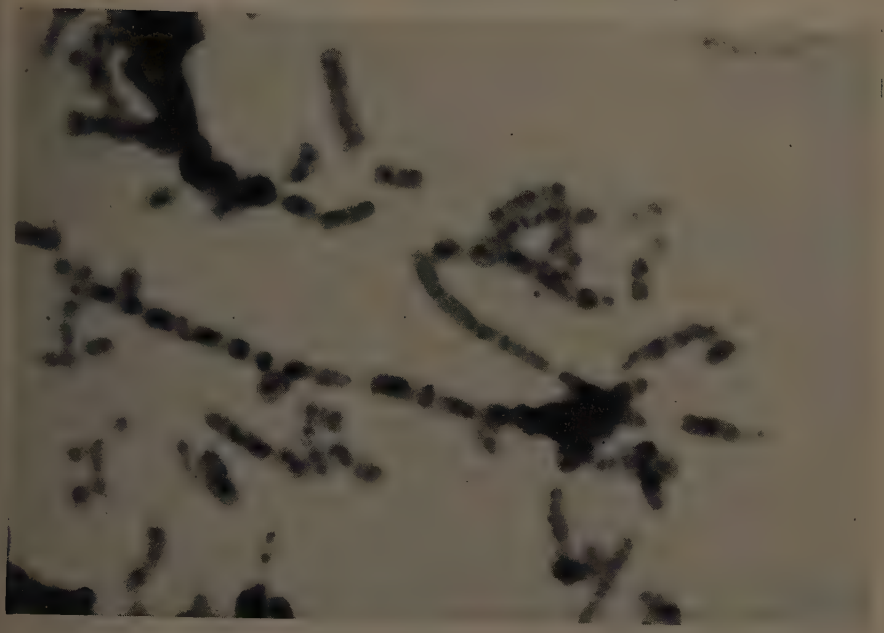


FIGURE 10. Conidia of *S. olivaceus* NRR L-B-1125. Adapted from work reported by Kinoshita and Itagaki.[12]

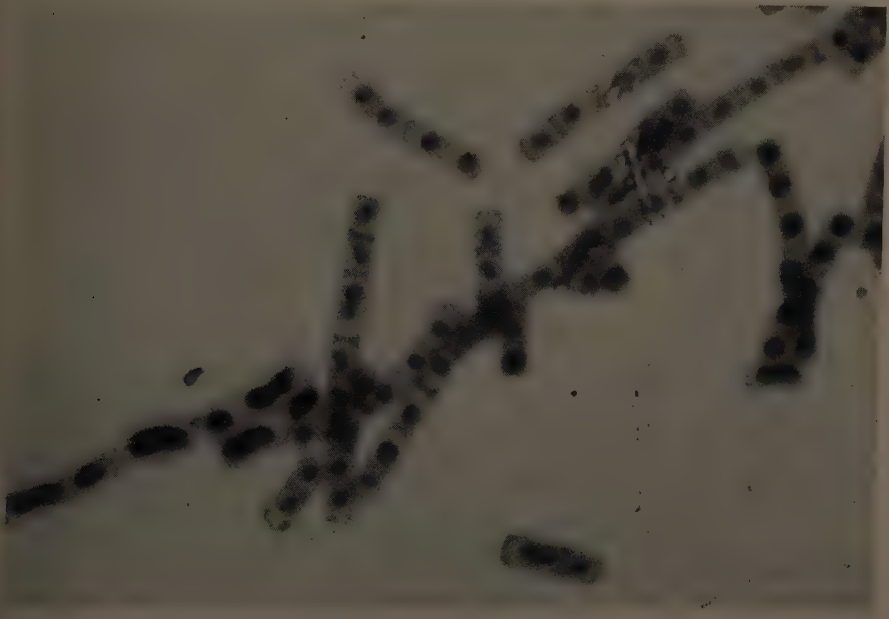


FIGURE 11. Conidia of *S. pheochromogenus* W-41. Adapted from work reported by Kinoshita and Itagaki.[12]

TABLE 7

DISTRIBUTIONS OF THE NUMBER AND SHAPE OF NUCLEI IN THE SPORES OF *STREPTOMYCES* SPP.*

Strains	Number of nuclei			Shape of nuclei				
	1	2	3	●	▬	●—●	● ●	Others
	Percentages							
Group A								
S. albogriseolus NRRL-B-1305	100	rare	—	100	rare	rare	rare	—
S. sp. NRRL-2288	100	—	—	100	—	—	—	—
S. coelicolor 9023	85	13	—	78	7	8	5	2
S. californicus ATCC-3312	100	—	—	100	—	—	—	—
S. flaveolus P-10	100	—	—	100	—	—	—	—
S. rimosus NRRL-2234	100	—	—	100	—	—	—	—
S. griseoflavus 142-305	96	4	—	87	9	2	2	—
S. flavovirens ATTC-3320	100	—	—	100	—	—	—	large nucleus
S. griseoluteus P-37	92.5	6.5	1	92	0.5	0.5	6	—
Group B								
S. roseochromogenus 0-36-738	?							
S. roseochromogenus B-2	?							
S. cinnamonensis 154-T3	100	rare	—	100	rare	rare	rare	—
S. flavochromogenes T349-15	100	—	—	100	rare	—	—	—
S. flavochromogenes H-3206	100	—	—	100	rare	—	—	—
S. antibioticus U-1091	100	—	—	100	—	—	—	—
S. viridochromogenes CBS (IFO-3113)	100	—	—	100	—	—	—	diffused nuclei
S. pheochromogenus 253	100	—	—	100	—	—	—	—
S. pheochromogenus S-66-B	100	—	—	100	—	—	—	—
S. pheochromogenus W-41	97	3	—	97	—	2.5	0.5	—
S. aureus ATCC-3309	100	—	—	100	—	—	—	—
S. erythrochromogenes W-115C	92	8	—	87	—	4	4	5
S. lavendulae ATCC-8664	83	17	rare	62	18	12	5	3
Group C								
S. microflavus 13-A	92	8	—	82	10	2.5	5.5	—
S. griseus No. 1	100	rare	—	100	—	rare	rare	—
S. griseus SX_2-O-11	100	—	—	100	—	—	—	—
S. griseus H-12	100	—	—	100	—	—	—	—
S. griseus TS-601	100	—	—	100	—	—	—	—
S. griseus SN-14	100	—	—	100	—	—	—	—
S. griseus MC-33	100	rare	—	100	rare	rare	rare	—
S. olivaceus NRRL-B-1125	85	12	3	78	7	4	8	3
S. olivaceus CR-74	98.5	1.5	—	96	2.5	—	1.5	comma-shaped nuclei, non-nucleate
S. olivaceus PD-60	100	—	—	100	rare	—	—	—
S. olivaceus G	100	—	—	100	—	—	—	—
S. olivaceus GSM^{r}_{5000}	?							
S. sahachiroi	95	5	—	95	—	5	rare	—
S. halstedii CBS(IFO-3199)	100	—	—	100	—	—	—	—
S. lipmanii 3331	100	—	—	100	—	—	—	—
S. fradiae 117	100	—	—	100	—	—	—	—
S. fradiae 3535	?	?	—	?	many	many	rare	—
S. fradiae (Okami)	100	—	—	100	—	—	—	—

Strains are classified in three groups according to the seventh edition of Bergey's *Manual of Determinative Bacteriology* (1957).

* Adapted from work reported by Kinoshita and Itagaki.

without actual separation of components just prior to conidial maturation, that is, by a sort of autogamy. However, the present data are still insufficient to indicate whether the nucleus is diploid or bipartite. It is also possible that the conidia are dikaryotic, and that some are destined to be diploid. This process may play a role in the sexual life cycle.

Genetic interaction that may involve mutagenesis as well as recombination may occur in heterokaryons when two kinds of nuclei come into contact with each other. Nuclear fusion may be the most probable step. The complete or incomplete zygote nuclei thus formed may segregate mostly into their components before maturation of the conidia, but possibly may have infrequent chances to migrate into the conidium before segregation. These hetero- or hemizygotes would segregate largely homozygous descendants, as observed in our experiments. However, these points should be subjected to further investigation.

Summary

One of 2 aspects of the cytogenic studies with *S. griseoflavus* has been concerned with genetic recombination in this organism. Recombination did occur in a mixed culture of 2 mutants, as determined on appropriate selective media. Every mutant employed was nutritionally deficient at 2 loci. Among the conidia examined from the mixed culture, 1 in 10^5 to 10^8 was shown to be nonparental by appropriate selection procedures. Six different intercrosses among 6 mutants were tested in this way. The most probable explanation for the recombination appears to be the incomplete fusion of nuclei, with subsequent segregation in the heterokaryotic hypha. The second phase in this study was an investigation of the status of nuclei in conidia. X-ray inactivation of the conidia gave a 2-hit survival curve, indicating the presence of 2 sets of hereditary units in each conidium. The immediate detection of biochemical mutants among conidia irradiated by ultraviolet light was not successful by the replica-plating technique. However, subsequent incubation on complete medium revealed a number of such mutants. This was explained on the basis of segregation delay. Furthermore, the colonies formed by reversion of auxotrophic mutants yielded both revertant and auxotrophic conidia, indicating a heterozygous origin. In cytological observations, conidia were found to be uninucleate, but the chromatinic substance in the immature conidium occasionally had 2 spheres. It seems likely that the nucleus in the conidium duplicates without actual separation of components, forming a bipartite or diploid nucleus just prior to maturation. However, it is possible that a small portion of the nuclei in conidia, produced by heterokaryons, is hetero- or hemizygotic. Such a condition of nuclei should be considered in mutation studies that involve the search for better antibiotic producers, as well as in the studies on genetic interaction. There are many species of *Streptomyces* that exhibit similar cytological behavior.

Acknowledgments

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VARIABILITY IN STREPTOMYCETES

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Classically, variations in living things can be produced by one or a combination of the following: environmental influences, genetic hybridization, and mutations. Variations in the actinomycetes have been the subject of numerous studies, with the result that many authors have accused them of being extremely unstable microorganisms. Lieske (1921), for example, wrote: ". . . dass die Strahlenpilze in bezug auf ihre morphologischen und physiologischen Eigenschaften eine so grosse Veränderlichkeit aufweisen, wie kaum eine andere Organismengruppe."* And again: "Die Strahlenpilze, die in fast allen morphologischen und physiologischen Eigenschaften äusserst labile sind, stellen jedenfalls ein sehr geeignetes Material für exacte vererbungstheoretische Versuche dar."†

Lieske studied colonial variations in more than 100 cultures for a number of years and, in an extreme example, isolated from one sectoring colony 6 variants that differed in odor, temperature requirements, diffusible pigment production, and presence, location, and color of spores. He was so extremely careful in maintaining his cultures, usually propagating them from single spores or filaments, that it is unlikely that these extreme variations could have arisen from mixed inocula. Lieske considered it possible that nuclear fusion might occur in filaments, but he did not think actinomycetes sexual, as are higher plants and animals.

The influence of the substrate on colonial variations was studied by Krainsky (1914), who emphasized the necessity of using synthetic media and controlled cultural conditions for reproducible colonial characteristics useful in classification.

Jones (1940, 1950) on the other hand, took a more moderate view of variations in actinomycetes, and has demonstrated the usefulness of colonial characteristics in taxonomy when synthetic media and standard cultural conditions are maintained. He considered most variations to be of a temporary nature, and maintained that permanent ones could be evaluated. After careful study of differences in a number of strains he concluded: "Nor is there any real problem of maintaining relatively constant strains; permanent variations are rare; the range and mean of fluctuating variations are determinable; the loss of characteristics through prolonged culture can be restored." Again, he said, "Relatively few isolates, in my experience, have a strong tendency to sector or to exhibit a wide range of fluctuating variations." Macroscopic markers of colonies that Jones (1950) considered of taxonomic importance included size, shape, margin, topography, and position of aerial mycelium, colony color, diffusible pigments, and propensity for sectoring. The influence of the penulti-

* ". . . the ray fungi exhibit a greater variability in their morphologic and physiological characteristics than almost any other group of organisms.

† "The ray fungi, which are extremely labile in almost all morphologic and physiological characteristics, present in any case, very suitable material for exact theoretical genetic experiments."

mate medium on these characteristics, reported by Jones (1946) and later by Erikson (1947), must be considered.

Variations in *Streptomyces* have been particularly vexing to the taxonomists of the group. Species or isolates of commercial value have posed difficult problems in this regard: for example, the decision as to whether to assign a new species name when deviations from named species were minor; just where the line between isolates, strains, varieties, and species should be drawn has depended largely on the exigencies of the situation and the judgment of the persons concerned. Many times it has been expedient to describe an isolate as new, rather than face the uncertainties of identification with descriptions of already published species. Backus *et al.* (1954) expressed this view: ". . . we consider regrettable any tendency for investigators to describe and establish new species in this already confused genus on the basis of only a few comparatively minor deviations from some well established species type, or as a result of observing the characteristics of a single isolate and without due consideration even of its inherent capacity to vary."

Burkholder and Sun (1954) proposed that a system of convenience be established with relatively few species groups to which strains having special characteristics could be assigned, with similar rank as horticultural varieties in fruit crops. They defined both strain and species in *Streptomyces* to meet the requirements of this concept.

Although tacitly inferred as a means of variation in *Streptomyces* by many authors, genetic recombination was reported first in the genus by Sermonti and Spada-Sermonti in 1955. Using the classic methods of producing and identifying biochemical mutants developed by Beadle and Tatum (1945) and the techniques of producing heterokaryons in imperfect fungi used so successfully by Pontecorvo (1946), these authors reported the production of stable prototrophs in *S. coelicolor* by growing together complementary artificially produced auxotrophs. From these mixtures prototrophs arose that were interpreted as recombinants of three types: (1) those having wild characters of both parental strains, (2) those having wild characters from one parent and mutant from the other, and (3) those having mutant characters from both parental strains (Sermonti and Spada-Sermonti, 1956). These latter investigators considered the presence of the third type as their most convincing evidence that recombinants had been demonstrated. Bradley and Lederberg (1956) produced auxotrophic mutants in *S. griseus* and *S. cyaneus*. Selected identified auxotrophs of *S. griseus*, when plated together, yielded prototrophs, spores from which produced only parentallike colonies. Thus, the findings of Bradley and Lederberg (in a different species) did not support those of Sermonti and Spada-Sermonti (1956), as stable prototrophs were not demonstrated. Bradley and Lederberg (1956) concluded that their results were due to heterokaryosis and that synkaryosis was not involved. They reported syntrophy between certain auxotrophs, but experimented only with those that did not exhibit this phenomenon. Heterokaryosis was confirmed by plating hyphal fragments that gave rise only to prototrophs. In the same paper these workers reported, for the first time, the detection of anomalous heterokaryons. These were of two kinds: one grew on doubly supplemented medium that was adequate for each parent, but not on any single supplement, and the

other required a third supplement. Both types of anomalous heterokaryons produced prototrophs when combined with parental strains. No explanation of these observations was offered at this time.

Szybalski and Braendle (1956) reported the production of prototrophs by combining auxotrophic mutants of *S. griseus*, spores from which again produced only parental types, thus agreeing with the findings of Bradley and Lederberg (1956). The former authors considered the conidia of *S. griseus* uninucleated and believed that these observations were best explained by heterokaryosis. They tested a number of species, including *S. coelicolor*, in which prototrophs from mixtures of auxotrophic mutants produced prototrophic conidia and "showed segregation of nonselected characters indicating gene recombination."

Braendle and Szybalski (1957*a*, 1957*b*) reported heterokaryosis in a number of other species of *Streptomyces*, including *S. griseoflavus*, *S. venezuelae*, *S. albus*, *S. sphaeroides*, and *S. fradiae*. The latter species was found to produce recombinants, but with a low frequency. A special method was used involving the prolonged cultivation of heterokaryotic mycelia and the selection of the rare conidia that produced recombinant colonies. *S. coelicolor* was reported to form recombinants much more frequently than *S. fradiae*. Szybalski (1957) summarized genetic interactions of *S. fradiae* as follows: "... in a heterokaryotic mycelium, nuclei undergo infrequent fusion, with the production of highly unstable diploid nuclei. During subsequent divisions, one chromosomal complement is progressively eliminated (haploidization) by a process distinct from orderly meiosis. Mitotic crossing-over often intercedes diploidization and haploidization. The comparatively high yield of prototrophs even on partially supplemented media, may be attributed to selective conditions which favor more rapid multiplication of prototropic nuclei in the heterokaryotic mycelium."

Bradley (1957) reported the detection of unstable prototrophs in *S. coelicolor* which produced parental types from spores, and evidence that the "presumptive recombinants which express the mutual phenotype of each parent actually contain the complete genome of both parents." Thus, he challenged the interpretation of Sermonti and Spada-Sermonti (1956) and Szybalski and Braendle (1956) that production of stable prototrophs from conidia was direct evidence for recombination.

Saito (1958) demonstrated heterokaryosis in *S. griseoflavus* by prolonged incubation of two auxotrophic mutants, from which only parental-type conidia were produced. He was also able to induce the production of recombinant colonies by mixing two double auxotrophs on a medium supplemented with limited amounts of nutrients. Conidia from these colonies were stable. Heterogeneous colonies were found (22 of 53) that produced conidia of one recombinant type together with either parental type and, in two colonies, two recombinant types and one parental were identified. Saito postulated that the former probably was due to formation of heterokaryons between the recombinant and parental mycelial growth, and the latter to "heterozygous and/or heterokaryotic conidia." He believed that genetic recombination occured in heterokaryotic mycelium prior to spore production, but that synapsis was doubtful in the light of imbalance of recoverable recombinants. Regarding nuclear behavior Saito (1958) stated: "With *S. griseoflavus* the conidium con-

sists of a diploid or bipartite nucleus as reported previously. However, the nuclei were homozygous regardless of the mixture of nuclei in the mycelial phase because duplication in the conidium was shown to result from self-replication of a single nucleus. Under these conditions it seems improbable that genetic recombination occurs within the conidia."

In a careful analysis of rarely produced anomalous heterokaryons in *S. coelicolor*, Bradley (1958) found that nonparental auxotrophs were of two types: (1) those with one nutritional requirement of each parent, and (2) those with nutritional requirements not previously recognized in either parent. He found that in the first type intact parental genomes were present, but in imbalanced ratios. After weighing the evidence, he concluded that the second type was probably due to mutations, as recombinations seemed improbable as the cause, since the large number of genes involved would require initial stocks to be excessively heterogeneous.

Hopwood (1957) reported the identification of all possible recombinants in a cross between two doubly auxotrophic mutants of *S. coelicolor*. These results were explained by the occasional fusion of nuclei in heterokaryons, followed by a reduction division.

The reports of compatibility systems in *S. coelicolor* by Bradley *et al.*, elsewhere in this monograph, and in *S. fradiae* by Szybalski and Braendle, elsewhere in this monograph, are notable advances in understanding and interpreting the genetics of streptomycetes. The existence of such systems may explain the low frequencies of heterokaryosis reported by these authors, especially in their earlier work. The finding of a thousandfold increase in frequency of heterokaryosis in *S. fradiae* when secondary parental strains were paired by the latter authors suggests that mating compatibility may be genetically controlled. That most sister conidia in a chain are of one parental type in *S. griseus*, but that both parental types are usually found in one chain in *S. fradiae* indicates different nuclear behavior during sporulation in these two species. If heterokaryosis is present and the conidia are uninucleated, both types should be present in single chains, relative numbers depending on the balance of parental nuclei. Results in *S. griseus* suggest strong imbalance of parental nuclei, if currently accepted views of nuclear behavior during sporulation are correct. Should it prove axiomatic that heterokaryons can be formed only between mutants of the same species, as reported by Braendle and Szybalski (1957b) for *S. fradiae*, a possibly useful criterion for determining relationships between strains would be established. It has been shown that hyphal fusions are produced only between the same species in certain dermatophytes, for example (Langeron and Vanbreuseghem, 1952). However, the presence of mating types might render this of limited value in *Streptomyces*.

The interpretation of results of genetic studies in *Streptomyces* is complicated by several factors, the most important of which are the paucity and inconclusiveness of cytological evidence, the difficulty of correctly identifying the phenotype of conidia, the necessarily long period between haploidization and scoring of recombinants, and the possible production of heterokaryons between parents and daughter recombinants. The apparent multiplicity of genetic interaction in these organisms presents evidence that autogamy, parasexuality, sexuality,

heterokaryosis, selective action, and mating types all may be involved. In view of these possibilities, various interpretations of findings by different investigators are not surprising. Everyone agrees that heterokaryons are produced by all species studied, and this production apparently is the principal mechanism involved in *S. griseus*. Nonuniform behavior of various strains and species may be due to different nuclear arrangements in conidia of various members of the genus. It has been known for a long time that the spores of certain imperfect fungi are heterokaryotic, whereas those of others are not (Hanson, 1938).

The mechanism of hyphal fusion probably furnishes one means of heterokaryosis, as the existence has been unequivocally demonstrated by Gregory (1956). Recombination appears to occur in species in which prototrophs derived from pairings of auxotrophic mutants may be propagated from conidia, although the mechanisms involved are subject to considerable speculative interpretation, and the findings of Bradley (1958) of intact genomes in apparent recombinants needs to be investigated more thoroughly.

Thus, using the basic techniques applied successfully to perfect and imperfect filamentous fungi, yeasts, and bacteria, the genetics of actinomycetes is beginning to be explored. Heterokaryosis and, possibly, recombination have been demonstrated in a few *Streptomyces* species. Fungi such as *Neurospora*, *Sordaria*, and *Schizophyllum* offer advantages of interpretation, as life cycles are known with certainty. *Streptomyces* shares with bacteria the uncertainties of nuclear behavior and the concomitant difficulty of making decisive observations. However, as exploration in this area is less than three years old, we should expect stimulation of investigations directed toward obtaining answers for such fundamental questions as: (1) is there an alternating haploid-diploid life cycle, (2) are conidia uni- or multinucleated, (3) are conidia diploid or haploid, and (4) what role may heterokaryosis play in the selection and evolution of the genus?

The genetic studies reported here offer an additional tool to the cytologist. As Jones (1954) so aptly emphasized: "The organism must be better understood before we can make any headway in comprehending variability in *Streptomyces*. First and foremost, an intensive cytological study is needed to give whatever direct information modern techniques can reveal on the finer structure of filaments and spores."

As Bradley (1958) has shown, the mutation rates of recessive characteristics of *S. coelicolor* suggest a haploid organism. However, this investigator has shown also that intact sets of genetic information from two parents are present in some conidia. This could be explained by the presence of bi- or multinucleated conidia, but most investigators who have considered the problem have concluded that they are uninucleated. Generally, hyphae have been reported to be coenocytic (Klieneberger-Nobel 1947, Webb *et al.*, 1954). If an analogy with fungi is permissible, most coenocytes produce coenocytic spores. The genetic suggestion that conidia of *Streptomyces* may be multinucleated finds support in cytological studies. Drechsler (1919), for example, said that with the ". . . appearance of one or several deep-staining granules within the spore" it ". . . seems not at all improbable, however, that . . . particularly those characterized by uniform size and moderate staining properties are nuclei."

Both divergent current views concerning the nuclear nature of streptomycetes have supporters. That there is a two-phase life cycle with haploid substrate mycelium and diploid aerial mycelium has been supported, at least in part, by Badian (1936), von Plotho (1940), Klieneberger-Nobel (1947), and McGregor (1954). Badian (1936) postulated that chromatin existed in hyphae as chromosomelike bodies that fused prior to conidia formation, so that each spore contained a bivalent chromosome. On germination the chromosome underwent two divisions, one of which was reductional, each germ tube receiving one of the four daughter chromosomes; von Plotho (1940) described Feulgen-positive bodies that concentrated and divided during conidial formation. Klieneberger-Nobel (1947) described an elaborate life cycle involving diploid conidia, which germinated to produce haploid substrate, or primary mycelium. The diploid phase was reestablished in initial cells formed from nests produced by the intertwining and fusion of special branches of the primary mycelium. The initial cells then produced diploid aerial mycelium, secondary mycelium, which segmented into the diploid conidia by septation. McGregor (1954) supported this general view, but found no evidence of nests in the strains he studied. Gregory (1956), although not primarily concerned with this problem, found no evidence of initial cells. He described swollen, partially germinated conidia that resembled the *Vierehyphensporen* of Lieske (1921) and the initial cells of Klieneberger-Nobel (1947) and McGregor (1954).

In contrast to the two-phase life cycle described above, we have the long-accepted view that streptomycetes are asexual organisms much as are the *Fungi imperfecti*, there being no essential difference between substrate and aerial mycelium. The substrate mycelium serves to absorb and transmit nutrients to the aerial mycelium, which is wider in diameter and mostly devoted to the production of conidia. This concept is supported by the finding of typical conidia in substrate mycelium produced in submerged culture by Carvajal (1947), who even found spores very similar to conidia produced in the germ tubes of conidia that were of aerial origin. However, different potentialities of substrate and aerial hyphae were demonstrated nicely by Erikson (1948) when she described the production of permanent variants (through ten subcultures) produced from conidia of *S. coelicolor*, while colonies from substrate mycelium produced only parental types. These findings might well be reexamined now that heterokaryosis and recombination have been reported in this species.

The inconsistencies of the cytological findings in streptomycetes emphasize the necessity for more careful cytological work, coupled perhaps profitably with genetic studies. Using the tools now available in improved staining procedures, phase-contrast microscopy, electron microscopy (especially with ultrathin microtomy), unequivocal cytological findings should be forthcoming.

Cytological and genetic findings in the presumably closely related, but relatively economically unimportant, genus *Nocardia* may be helpful, as there is evidence that this genus offers unexcelled material for such investigations. Studies to date are not so voluminous as for the genus *Streptomyces* and, consequently, not so contradictory. Cytological findings have been confined principally to my own (McClung, 1950, 1955, 1956), mostly of an exploratory nature in which distribution, size, shape, and behavior of chromatinic bodies are

deduced from studies by light, electron, and phase-contrast microscopy. Morris (1951) who proposed a complex life cycle involving a very short diploid phase, and Webb *et al.* (1954), Webb (1956), Clark and Webb (1957), and Webb and Clark (1957) presented radiobiological and cytological evidence for a diploid coccoidal stage in *N. corallina*, and described a life cycle summarized as follows: "... diploid coccoidal stage gives rise to a coenocytic diploid hyphal stage which fragments through a nuclear reduction division to form haploid binucleated bacillary cells. The bacillary cell nuclei fuse and the cell divides to form diploid coccoids." The report of Webb (1956) of recombination from crosses between white rough mutants and red smooth mutants, is the first genetic study in the genus, insofar as I am aware.

Therefore, we see that problems concerning variability in streptomycetes are far from settled. However, the genetic evidence presented in this monograph promises a means of understanding the genus *Streptomyces* in a way that we have not had before. There is good evidence that all three factors capable of producing variations in organisms are at work here. In addition to environmental effects and spontaneous mutations, heterokaryosis most certainly, and recombination probably, play a role. Correct interpretations and resolution of the genetics of *Streptomyces* will require the best efforts of geneticists and cytologists alike because of the apparent multiplicity of genetic interactions. The extent to which man will succeed in breeding *Streptomyces* tailored to fit specific requirements will depend on the extension of genetic studies in the genus. Since these studies are not more than three years old, we have every reason to be optimistic of a successful resolution; meanwhile, basic microbiology will have profited also.

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LINKAGE AND THE MECHANISM OF RECOMBINATION IN *STREPTOMYCES COELICOLOR**

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Introduction

Genetic recombination has been reported to occur in several species of *Streptomyces*: *S. coelicolor*[1-4], *S. fradiae*[4], *S. rimosus*[5], and *S. griseoflavus*.[6] Other authors have not drawn definite conclusions on linkage. It is the object of this paper to show that quantitative data on recombination in a strain of *S. coelicolor* can be obtained and used to draw conclusions on linkage and on the mechanism of recombination.

Preliminary experiments with auxotrophic and streptomycin-resistant mutants demonstrated the existence of a rare process of genetic recombination in the two strains of *S. coelicolor* studied.[3] First crosses were made on a minimal medium only partially supplemented, in order to increase the ratio of recombinant to nonrecombinant progeny. Crosses on this medium yielded several genotypes of recombinants, including some combining nutritional deficiencies from both parents, but the majority were prototrophic. Results thus resembled those of Sermonti and Spada-Sermonti,[2] who used a similar experimental procedure with a different strain of *S. coelicolor*.

The possibility that prototrophic recombinants were heterozygous diploids rather than true recombinants was investigated. A comparison of the dose-survival curves during X irradiation of such isolates with those of the parents gave no evidence of diploidy. A search for auxotrophic segregants from these isolates suggested that they were very stable and so were unlikely to be an intermediate stage in the origin of other recombinant genotypes. It was concluded that prototrophs formed the major class of recombinant, either because of the particular linkage relations of the parental markers or, more probably, since several combinations of markers gave the same result, because prototrophic nuclei had a selective advantage on supplemented minimal medium and reproduced more rapidly than other genotypes. In order to obtain information on linkage it was essential to minimize such selective effects. An approach to this problem, using *S. coelicolor* strain A3(2)†, is outlined below.

Detection and Estimation of Linkage

The technique and results of crosses will be described fully elsewhere. The most important points of procedure were the following.

(1) Parents differed in 4 selected markers. This is the minimum number capable of providing information on the equality of complementary genotypes, and the maximum consistent with avoiding gross differential viability effects.

(2) Crosses were made on a complete medium, in order to minimize the selective advantage of certain genotypes.

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† This isolate was obtained through the courtesy of D. Erikson. It was derived by her[7] from one of the agar-decomposing strains (Waksman's 3443) studied by Stanier.[8]

(3) Spores were harvested from crosses soon after sporulation began (3 to 5 days) so as to reduce the time available for differential multiplication of different genotypes.

(4) Recombinants were recovered on the minimal medium used by Sermonti and Spada-Sermonti,[1] the appropriate amino-acid supplements being added at the rate of 50 mg. of the L-acid/l.

With 4 loci segregating, 16 classes of progeny were possible, in 8 complementary pairs. The maximum number of classes was recovered by using 4 differently supplemented selective media, on each of which 4 recombinant classes

TABLE 1

RESULTS OF CROSS A27: me-2 phe-1 st-1^r × his-1 st-1^s

Genotypes of recombinants				Supplements added to selective medium								Average estimates of recombinant frequencies
				Histidine, phenylalanine, and streptomycin		Methionine		Histidine, methionine, and streptomycin		Phenylalanine		
his	me	phe	st	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	
+	+	+	r	1	3.6	0	0	4	6.3	6	1.9	2.9
+	+	+	s	—	—	0	0	—	—	22	7.1	3.6
−	+	+	r	13	46	—	—	46	71	—	—	58
+	−	+	r	—	—	16	64	46	71	—	—	68
+	−	+	s	—	—	74	296	—	—	—	—	287
−	+	−	r	79	278	—	—	—	—	—	—	
+	+	−	r	0	0	—	—	—	—	24	7.7	3.9
+	+	−	s	—	—	—	—	—	—	4	1.3	0.7
−	−	+	r	—	—	—	—	0	0	—	—	
Totals				93	327	90	360	96	149	56	18	

The genotypes of the nine classes of recombinants for which selection can be made are given, together with the compositions of the four selective media. Column *a* contains the numbers of recombinants of each genotype in the sample of recombinants isolated from each selective medium, the total for this column being the size of the sample. Column *b* contains the frequencies of each recombinant genotype appearing on each selective medium per unit volume of spore suspension plated. These are calculated from the figures of column *a* and the average recombinant count per plate, the latter appearing as the total for column *b*. Averages of the figures in the four columns *b* give the average estimates of recombinant frequencies.

could grow. In this way, at least 1 member of each of the 7 complementary pairs of recombinant progeny could be recovered, with *both* members of 2 pairs. The relative frequencies of the different genotypes were obtained as follows. The total number of recombinants appearing on each selective medium per unit volume of spore suspension was determined by plating at suitable dilutions and counting the number of colonies per plate. The proportions of each of the 4 possible genotypes contributing to the total recombinant count on each medium were determined by isolating a random sample of recombinants and classifying them by replica plating to diagnostic media. The frequency of each class of recombinant per unit volume of the original spore suspension could then be calculated. The results of a typical cross are given in TABLE 1.

Two conclusions can be drawn from the results of a large series of crosses:

(1) Since there was generally good agreement between estimates of the frequency of a given genotype recovered on different selective media. Moreover, the ratio of colony-forming to total spores plated on each medium remained constant at different densities of plating. Thus competition effects, as found by Grigg[9] in *Neurospora*, seem to be largely absent.

(2) Differences between estimates of the frequencies of members of complementary pairs of genotypes were rarely greater than those between estimates of the frequency of the same genotype on different media. Thus complementary genotypes appear to be present in equal numbers. This means that an estimate of the frequency of only on member of each pair of complementary genotypes is required in order to determine the relative frequency of all cross-over patterns.

In TABLE 1, the 7 average estimates of recombinant frequencies correspond to the relative frequencies of the 7 patterns of cross-over between the 4 loci; only the eighth, noncross-over class, cannot be estimated. From these 7 figures, the relative frequencies of recombination between the 6 pairs of loci can be calculated by adding them in appropriate groups of 4 (TABLE 2). Since the

TABLE 2

RELATIVE FREQUENCIES OF RECOMBINATION BETWEEN PAIRS OF LOCI IN CROSS A27

Pairs of loci	Relative frequency of recombination	Recombination fraction
his-1 me-2	11	0.02
phe-1 st-1	130	0.2
his-1 phe-1	362	0.5
his-1 st-1	349	0.5
me-2 phe-1	360	0.5
me-2 st-1	352	0.5

size of the parental class is unknown, recombination fractions cannot be calculated directly. However, since the same (maximum) frequency of recombination is shown between members of 4 of the pairs of loci, this figure can be taken to represent a recombination fraction of 0.5. The frequency of recombination between members of the remaining 2 pairs of loci therefore can be expressed relative to the average for the other 4, giving the figures in the right-hand column of TABLE 2. Thus, we can tentatively assign the 4 loci to 2 linkage groups, 1 containing me-2* and his-1, with a recombination fraction of the order of 0.02, the other containing phe-1 and st-1, with a recombination fraction of the order of 0.2.

That the calculated frequencies of the different genotypes of recombinant reflect primarily the linkage relations of the markers and not merely the relative viability or rate of asexual multiplication of genotypes is shown by the results of crosses containing the same 4 markers in different coupling arrangements. Only 3 of the possible 8 crosses can be used, since each parent must contain 2 markers for which selection can be made. An example of such a set of 3 crosses is given in TABLE 3, and the results of the crosses in TABLES 4 to 6.

* The explanations of all symbols are given in the caption to FIGURE 1.

Considering the data in the same way (TABLE 7) as for cross A27 above, it appears that the most likely arrangement of the 4 loci segregating in this set of crosses is that me-2, his-1, and arg-1 are linked, in that sequence, but are unlinked with st:

(me-2 his-1 arg-1) st

Recombination regions *a* *b* *c*

The results of the 3 crosses are summarized in TABLE 8 on the basis of this arrangement. The genotypes resulting from the 7 patterns of cross-over, to-

TABLE 3

SET OF THREE CROSSES CONTAINING THE SAME FOUR MARKERS IN DIFFERENT COUPLING ARRANGEMENTS

Cross number	Parental genotypes
A42	me-2 his-1 st-1^r × arg-1 st-1^s
A54	his-1 arg-1 st-1^r × me-2 st-1^s
A55*	me-2 arg-1 st-3^r × his-1 st-3^s

* Since st-1 and st-3 behave as alleles, this cross can be regarded as containing the same markers as the other two.

TABLE 4

RESULTS OF CROSS A42*: me-2 his-1 st-1^r × arg-1 st-1^s

Genotypes of recombinants				Supplements added to selective medium								Average estimates of recombinant frequencies
				Histidine, arginine, and streptomycin		Methionine		Methionine, arginine, and streptomycin		Histidine		
me	his	arg	st	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	
+	+	+	r	6	6.0	20	4.6	3	2.4	19	4.4	4.4
+	+	+	s	—	—	76	17	—	—	66	15	16
+	−	+	s	—	—	—	—	—	—	8	1.8	1.7
−	+	−	r	—	—	—	—	2	1.6	—	—	
+	−	+	r	0	0	—	—	—	—	3	0.7	0.4
−	+	+	s	—	—	0	0	—	—	—	—	0
+	−	−	r	0	0	—	—	—	—	—	—	
−	+	+	r	—	—	0	0	0	0	—	—	0
+	+	−	r	87	86	—	—	90	71	—	—	79
Totals				93	92	96	22	95	75	96	22	

* Data are presented as in TABLE 1.

gether with their frequency relative to the class resulting from cross-over in region *c* are given for each cross. In general, the genotypic frequencies vary from cross to cross in the way that would be expected from the coupling arrangements of the parental markers. There is a certain amount of discrepancy between crosses, some of which, at least, must be due to viability disturbances. The equality of cross-over patterns *a* and *a-c* and of *b* and *b-c* reflects the absence of linkage of st with me-2, his-1, and arg-1. If the excess of *a-b* over *a-b-c* is genuine, it might suggest that st is, in fact, linked to the other markers in such

a way that there is interference between regions *a* and *b* and region *c*, but sufficiently far away to give 50 per cent recombination with them. The arrangement of loci assumed above is clearly the most probable, with recombination

TABLE 5

RESULTS OF CROSS A54*: his-1 arg-1 st-1^r × me-2 st-1^s

Genotypes of recombinants				Supplements added to selective medium								Average estimates of recombinant frequencies
				Methionine, arginine, and streptomycin		Histidine		Histidine, methionine, and streptomycin		Arginine		
me	his	arg	st	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	
+	+	+	r	1	0.3	4	1.4	7	2.8	38	1.2	1.4
+	+	+	s	—	—	8	3.2	—	—	44	1.4	2.3
+	−	+	s	—	—	30	11	—	—	—	—	
−	+	−	r	21	7.2	—	—	—	—	—	—	9
+	−	+	r	—	—	50	18	18	7.3	—	—	13
−	+	+	r	73	25	—	—	71	29	—	—	27
+	+	−	s	—	—	—	—	—	—	1	0.03	
−	−	+	r	—	—	—	—	0	0	—	—	0.02
+	+	−	r	1	0.3	—	—	—	—	9	0.3	0.3
Totals				96	33	92	33	96	39	92	3	

* Data are presented as in TABLE 1.

TABLE 6

RESULTS OF CROSS A55*: me-2 arg-1 st-3^r × his-1 st-3^s

Genotypes of recombinants				Supplements added to selective medium								Average estimates of recombinant frequencies
				Histidine, arginine, and streptomycin		Methionine		Histidine, methionine, and streptomycin		Arginine		
me	his	arg	st	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	
+	+	+	r	0	0	0	0	0	0	3	0.7	0.2
+	+	+	s	—	—	3	2.5	—	—	28	6.9	4.7
+	−	+	r	80	83	—	—	80	68	—	—	75
−	+	+	s	—	—	72	59	—	—	—	—	
+	−	−	r	13	13	—	—	—	—	—	—	37
−	+	+	r	—	—	18	15	12	10	—	—	13
+	+	−	s	—	—	—	—	—	—	25	6.2	
−	−	+	r	—	—	—	—	3	2.6	—	—	4.4
+	+	−	r	1	1.1	—	—	—	—	25	6.2	3.7
Totals				94	97	93	77	95	81	81	20	

* Data are presented as in TABLE 1.

fractions in regions *a* and *b* of the order of 0.04 and 0.2, respectively, the recombination fraction in region *c* being taken to be 0.5.

Linkage Groups of S. coelicolor A3(2)

Results obtained by the procedure described in the previous section pointed to the existence of 2 linkage groups in *S. coelicolor* A3(2), each containing 3

markers (these markers are indicated by arrows in FIGURE 2). With certain markers located, linkage of these with new markers was detected by determin-

TABLE 7

RECOMBINATION BETWEEN PAIRS OF LOCI IN THE CROSSES OF TABLE 3

Pairs of loci	Crosses					
	A42		A54		A55	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
me-2 his-1	2.1	0.01	4.0	0.05	13	0.06
me-2 arg-1	23	0.13	26	0.33	58	0.26
his-1 arg-1	20	0.11	22	0.28	55	0.25
me-2 st	84	0.5	38	0.5	116	0.5
his-1 st	85	0.5	38	0.5	121	0.5
arg-1 st	97	0.5	41	0.5	93	0.5

For each cross, column *a* gives the relative frequencies of recombination between pairs of loci; column *b*, the recombination fraction obtained by expressing the figures of column *a* for the first three pairs of loci relative to the average for the last three, and putting the latter equal to 0.5.

TABLE 8

RELATIVE FREQUENCIES OF THE GENOTYPES PRODUCED BY DIFFERENT CROSS-OVER PATTERNS IN THE CROSSES LISTED IN TABLE 3

Cross-over in regions	Crosses						Average frequencies
	A42		A54		A55		
	Genotype*	Frequency	Genotype*	Frequency	Genotype*	Frequency	
c	+ + − r	100	− + + r	100	+ − + r	100	100
a	+ − + r	0.5	+ + + s	8.5	+ + − r	4.9	4.6
		1.3		6.9		5.4	4.5
a-c	+ − + s − + − r	2.1	+ + + r	5.3	+ + − s − − + r	5.9	4.4
b	+ + + r	5.5	+ − + s − + − r	33	− + + s + − − r	49	29
		3		40		34	29
b-c	+ + + s	21	+ − + r	47	− + + r	17	28
a-b	− + + r	0	+ + − r	1.1	+ + + s	6.2	2.4
		0		0.5		3.2	1.2
a-b-c	− + + s + − − r	0	+ + − s − − + r	0.06	+ + + r	0.2	0.1

* Genotypic formulas give the alleles in the sequence: me-2, his-1, arg-1, st.

ing the recombinant count per plate in crosses involving old and new markers, without isolating and classifying recombinants. Each plating medium selects only for recombination between a pair of loci, so that the recombinant count is a measure of the frequency of recombination between them. As an example,

a cross containing a new marker, me-3, gave the results of TABLE 9. Arg-1 and st-1 are known to give 50 per cent recombination, so that a count of 430 corresponds to this figure. Me-3 is clearly linked with phe-1, but not with arg-1; the known linkage of phe-1 with st-1 is confirmed.

TABLE 9

CROSS A186: arg-1 phe-1 st-1^r × me-3 st-1^s

Supplements added to selective medium	Average recombinant count per plate	Loci between which recombination is selected
Methionine, phenylalanine, and streptomycin	430	arg-1 st-1
Arginine, methionine, and streptomycin	219	phe-1 st-1
Phenylalanine	440	arg-1 me-3
Arginine	109	phe-1 me-3

	I					II							III
	am-1	me-2	his-1	arg-1	arg-4	ser-1	hs-1	his-4	thre-1	st-1	me-3	cys-6	phe-1	phe-3	cys-5
am-1	+	+	−	o	o		o	−	−	−	−	o	−	−	−
me-2	+	+	+	+	±		o	−	−	−	−	o	−	−	o
his-1	+	+	±	±			−	−	−	−	o	−	−	o	−
arg-1	+	+	+				−	−	−	−	−	−	−	−	−
arg-4	+	+					o	−	o	o	−	o	−	−	−
ser-1							o	−	−	−	−	o	−	−	−
hs-1	+	+	±	±	o	−	−	−							
his-4	+	+	±	±	−	o	−								
thre-1	o	+	o	o	−	−									
st-1	+	+	±	−	−										
me-3	+	+	±	−	−										
cys-6	+	±	−	−											
phe-1	+	−	−												
phe-3	−	−													

FIGURE 1. The results of tests for linkage between fifteen markers in *Streptomyces coelicolor* strain A3(2). Roman numerals refer to linkage groups. Key: +, close or moderately close linkage; ±, loose linkage; −, no linkage; 0, not tested. Marker abbreviations: am, amination deficient; me, methionine; his, histidine; arg, arginine; ser, serine; hs, homoserine; thre, threonine; cys, cysteine; phe, phenylalanine; st, streptomycin resistance. Numbers incorporated in marker abbreviations are the occurrence numbers of the mutants used in the crosses.

It was desirable to test for linkage in this way between all possible pairs of markers in order to be certain that viability disturbances were not leading to spurious results. Fifteen markers have been used, and at least 1 cross between most of the possible pairs has been made. Results are summarized in FIGURE 1.

They are highly self-consistent if the markers within linkage groups are arranged in the order shown. The 2 original linkage groups remain unlinked with each other, while a third contains a single marker. The probable order of loci and their approximate spacing within linkage groups are indicated in FIGURE 2.

Linkage groups I and II are each more than 50 units long. Since the 3 groups give 50 per cent recombination with each other, the total genetic map must, therefore, be more than 200 units, if all the loci are, in fact, borne on a single physical structure.

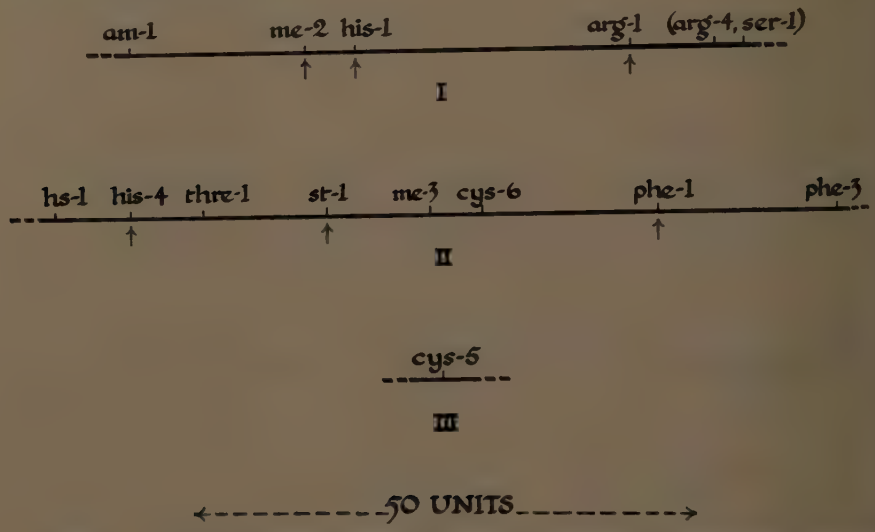

FIGURE 2. The three linkage groups at present recognized in *Streptomyces coelicolor* strain A3(2). Loci are distinguished by the occurrence numbers of the mutants used in the recognition of the loci; locus numbers have not been assigned. Arrows indicate the six markers located by the method described in the section entitled *Detection and Estimation of Linkage*. The order of arg-4 and ser-1 relative to the other markers is not yet known

Mechanism of Recombination

Since complementary recombinant genotypes appear to arise in equal numbers in crosses of *S. coelicolor* A3(2), genetic material probably is contributed equally by the 2 parents. It is possible, therefore, that recombination occurs between 2 complete parental genomes. Recombination between a whole genome of 1 parent and a partial genome of the other cannot at present be ruled out but, if this occurs, there must be approximately equal numbers of the 2 reciprocal types of encounter.

If we assume the dominance of streptomycin sensitivity, as in other strains,[10, 11] very few recombinants should have appeared on media containing streptomycin if the plating units had been heterozygous for streptomycin resistance. Since the frequencies of a given streptomycin-resistant genotype recovered on media with and without streptomycin were the same, postplating segregation does not appear to have occurred to an appreciable extent in crosses of this strain.

There is at present no evidence of a persistent diploid stage as a precursor of recombinants in *S. coelicolor* A3(2) although, in addition to the suggestion of Sermonti and Spada-Sermonti discussed below, such a stage has been postulated in *S. fradiae*.[4] It is not clear at the moment how diploidy in a bacterium would differ from heterokaryosis. Electron micrographs[12] of thin sections of mycelium and spores of *S. coelicolor* A3(2) reveal that this organism resembles other bacteria in the appearance of its nuclear material and in the apparent absence of nuclear membranes. As in other bacteria, it is not yet possible to say whether the nuclear material lies in a region discrete from the rest of the cell. If not, diploidy would be distinct from heterokaryosis only if the associated division of two genomes occurred, with the keeping together of the products of division, rather than the independent multiplication of each genome, as in a heterokaryon. It is important in this connection to discover whether the three linkage groups so far apparent in *S. coelicolor* A3(2) are really parts of a single genetic unit, since the existence of more than one physical unit would make ideas on the structure and mode of division of the nuclear material necessarily more complicated.

It is possible that no intermycelial event other than occasional hyphal fusion, leading to the establishment of heterokaryosis, is responsible for bringing the parental genomes together. Recombinants then might arise by, for example, a copy-choice mechanism, during the pairing of two genomes. Such a pairing process might be a normal part of the cycle of development, recombinants not arising, however, unless the mycelium were heterokaryotic; or alternatively a specialized mechanism leading to recombination might exist. The possibility of a special structure, such as a fusion cell, as the site of recombination remains. It is clear, however, that such a structure is not an essential part of the cycle of development from spore to spore in *S. coelicolor* A3(2), since simple side branches of the mycelium developed from the germination tubes of a single spore can sporulate.[13] The only more or less regular process apparent in the life cycle is, in fact, the delimitation of chains of spores. Since it is possible to select certain recombinant genotypes at the expense of others by crossing on supplemented minimal instead of complete medium, it is unlikely that segregation occurs during sporulation, with the immediate products of segregation entering the spores.

Discussion

It is clear from the results outlined that preliminary information on linkage in *S. coelicolor* A3(2) can be obtained. Differential viability, and what formally amounts to the same thing, differential multiplication of genotypes, cannot be avoided completely. By taking suitable precautions, however, they can be reduced to the point where they do not prevent the construction of tentative linkage maps.

Bradley,[11] working with a different strain of *S. coelicolor*, came to different conclusions. He found that mixed growths of two doubly auxotrophic strains did not give rise to true recombinants, but only to prototrophic heterokaryons. These were comparatively stable, only occasionally producing parental segregants and, even more occasionally, "anomalous heterokaryons." The latter required at least one of the growth factors needed by each parent. They did not

behave as true recombinants since, although comparatively stable, they segregated colonies showing the original parental phenotypes, as well as occasional prototrophic colonies. They were interpreted as heterokaryons in which particular ratios of the two parental types of nucleus gave rise to auxotrophic phenotypes. Bradley concluded that, owing to the complications caused by the occurrence of these different types of heterokaryon, quantitative data on recombination in this strain could not be obtained, so that linkage maps could not be constructed.

There seems little doubt that the situation described here in crosses of *S. coelicolor* A3(2) differs from that described by Bradley. In particular, the phenotypes of the majority, at least, of the selected progeny correspond to the genotypes of true recombinants. The following facts support this belief:

(1) Provided that differential multiplication was minimized, prototrophs did not form the major class of selected progeny except when the coupling of parental markers made them the major recombinant class.

(2) Recombinants isolated from crosses were stable; except for very rare reversions for single markers at a time, they did not give rise to any other prototrophic or auxotrophic phenotypes when grown separately.

(3) A given pair of complementary genotypes always gave the same pattern of recombinants when crossed together, whether they had been prepared from the wild type by successive mutation or had been isolated as recombinant progeny from a previous cross.

(4) When the same pair of complementary genotypes had been isolated from crosses involving different pairs of genotypes as parents, they gave the same pattern of recombinants when crossed; the progeny did not tend to resemble their grandparents as they should have done if their own parents had been merely heterokaryons of two types of nucleus. Thus, the parents used in Cross A54 were progeny from a cross of the genotypes me-2 arg-1 st-1^{r} and his-1 st-1^{s}, yet these two genotypes did not preponderate in the progeny of Cross A54 (TABLE 5).

As mentioned above, segregation appears to have been largely complete before plating of the progeny of crosses of strain A3(2). In another strain of *S. coelicolor*, Braendle and Szybalski[10] and Sermonti and Spada-Sermonti[14] found that some colonies produced by plating the progeny of a cross on selective media gave rise to more than one type of colony on subculture. Sermonti and Spada-Sermonti[14] dismissed as improbable the origin of such mixed colonies from more than one conidium or from a heterokaryotic conidium, and suggested that the plating units sometimes contained a nucleus that was heterozygous, at least for some markers, which later segregated. It would be difficult to eliminate completely the possibility of heterokaryotic plating units, since even if hyphal fragments, as such, were absent from a spore suspension, a proportion of conidia, probably varying from strain to strain, might be heterokaryotic. Phase-contrast studies on strain A3(2)[13] suggest that a population of conidia can be a rather heterogeneous collection of subunits developed from the mycelium, very different from, for example, the conidia of many Ascomycetes, with their uniform structure and regular development. Heterokaryons, in fact, appeared to be perpetuated regularly by single spores in Bradley's strain of *S. coelicolor*.[15] Thus, if heterokaryosis is indeed a preliminary to recombina-

tion, the postplating origin of recombinants might be expected to result ockaryotic plating units, the possibility should be considered that a recombinant colony developing amidst a dense background of parental conidia might stimulate their development syntrophically, re-establish a heterokaryon, and undergo a second cycle of recombination, with the possible production of a variety of genotypes.

It seems, therefore, that there are at the moment certain apparent discrepancies between the results obtained by different workers using different strains of *S. coelicolor.* How far these can be resolved remains to be seen, but it may be that strains referred to as *S. coelicolor* in fact form a rather heterogeneous group of organisms, so that genuine strain-to-strain differences in behavior might not be unexpected. Stanier[8] found that the strain from which A3(2) was derived (Waksman's 3443) resembled authentic *S. coelicolor* (Waksman's 3355) in many respects, although differing from it by agar decomposition and nonutilization of lactate. It is possible that A3(2), which appears at present to be more favorable for the study of genetic linkage than the strains investigated by Sermonti and Spada-Sermonti and by Bradley, may be related less closely to these strains than they are to each other.

Summary

Genetic recombination has been studied in *Streptomyces coelicolor* strain A3(2). By taking suitable precautions, quantitative data, making possible at least approximate mapping, have been obtained. Fifteen markers have been located so far in 3 linkage groups, containing 1, 6, and 8 loci, respectively. Complementary recombinant genotypes appear to be present in equal numbers among the progeny of crosses. There is no evidence for a diploid stage sufficiently prolonged to be detected. Results obtained with this strain are compared with those obtained by other workers using different strains of *S. coelicolor.*

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MECHANISMS CONTROLLING VARIATION IN STREPTOMYCETES*

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The gross appearance of an organism is the product of many interactions among its cytoplasm, nucleoplasm, and environment. Changes in the nutritive substrate, consequently, can provoke striking transient variations in colonial morphology: (1) some streptomycetes, for example, do not form aerial mycelia on complex medium, but sporulate well on synthetic medium;[1] (2) many streptomycetes synthesize dark-colored pigments during growth on complex substrates, but not while growing on synthetic substrates;[2] (3) the litmuslike pigment produced by wild-type strains of *Streptomyces coelicolor* is red in acid medium and blue in alkaline medium.[3] Most assuredly, slight alterations in the environment affect greatly the visible characteristics of an organism. Moreover, the morphology of colonies formed on replating is influenced by the composition of the growth medium of the inoculum.[4] Standardized cultural conditions, therefore, are necessary prerequisites for genetic and systematic studies.[5] Because the types and extent of variations encountered in the streptomycetes have been reviewed and discussed recently by several other workers, no additional introductory remarks are included here.[6-8]

Glossary

Phenotype: the sum total of the measurable traits that characterize an organism.
Genotype: the hereditary constitution of an organism resulting from its particular combination of genes.
Penetrance: the regularity with which a gene produces its effect in a population.
Expressivity: the extent or degree to which a particular trait is manifested in an organism.
Prototroph: an organism that grows on synthetic medium.
Auxotroph: an organism that requires one or more added supplements in order to grow.
Recombination: the process by which strains phenotypically unlike either parent are formed by intracellular synergism; recombination between two differently marked strains does not connote any particular interpretation.
Heterokaryosis: genetic cooperation of diverse nuclei within a common cytoplasmic field.
Heterozygote: an individual having a nucleus in which members of at least one pair of genes are dissimilar.
Segregation: the process by which contrasted Mendelian characters reappear in the offspring of heterozygotes.
Dissociation: any process by which variants arise from a clone as colonies and sectors.
Clone: a population derived from a single spore or hyphal segment.
Genome: a complete set of hereditary information.

Literature Survey

Until recently, the streptomycetes have been considered asexual microorganisms.[9] In 1955, however, Sermonti and Spada-Sermonti found that pairs of complementary, growth factor-dependent mutants grown in combination yielded spores that were able to grow on minimal medium. In addition, they dispensed spores from mixed cultures of two complementary, doubly auxo-

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trophic mutants onto minimal medium supplemented with a growth factor required by one parent and a growth factor required by the second parent. Infrequent growth factor-dependent recombinants, as well as prototrophic colonies, were isolated on the supplemented medium. The Sermontis interpreted their results as evidence for genetic recombination.[10] Subsequently, these workers unsuccessfully attempted to recover dissociates or segregants from recombinant clones. On these bases they concluded that the formation of new, stable prototrophic clones resulted from genic recombination between two auxotrophic clones.[11]

Lederberg and I, using techniques similar to those of the Sermontis, obtained prototrophic recombinants from mixed cultures of auxotrophic pairs of *S. griseus*.[12] Recombinants of *S. griseus*, unlike those of *S. coelicolor*, produced spores yielding one or the other parental type; moreover, individual hyphal segments from prototrophic recombinant mycelia were found to contain both of the intact parental genomes. The recombinants formed by pairs of *S. griseus* mutants, therefore, were heterokaryons.

Results obtained in my laboratory with *S. coelicolor* have confirmed the Sermontis' findings that two growth factor-dependent mutants grown in combination produced prototrophic recombinant colonies. Unlike the Sermontis, we recovered rare parental dissociates during serial subcultures of spores from recombinants.[13] In addition, dissociates were obtained that required initially a supplement needed by the first parent and a supplement needed by the second parent. The nonparental, auxotrophic dissociates reverted invariably to prototrophs or to one or the other parental types during subsequent subculture.

Using the Sermontis' strains of *S. coelicolor*, Braendle and Szybalski confirmed fully the results reviewed above.[14] Braendle and Szybalski additionally carried out reciprocal crosses employing streptomycin susceptibility as an unselected marker. They found that the genes controlling streptomycin susceptibility and the genes controlling glutamate synthesis were inherited as a unit. These workers interpreted their data as evidence of nuclear fusion, whereby a transient heterozygous diploid nucleus that ultimately yielded haploid segregants was formed. Unfortunately, the two streptomycin-resistant strains were not crossed to determine if these genes were allelic. Because different genes, one dominant and the other recessive, may be involved, their conclusions are not definitively established.

Alikhanian, using *S. rimosus*,[15] Szybalski with *S. fradiae*[16], and Ikeda and Saito with *S. griseoflavus*[17], recently have reported results similar to those obtained with *S. coelicolor*.

Genetic Composition of Conidiospores

On the basis of cytological studies, conidiospores of most streptomycetes have been considered uninucleate[14, 18] (see Saito and Ikeda, elsewhere in this monograph). Contrariwise, some strains have been reported to form multinucleate spores.[21] Genetic data obtained in my laboratory have been consistent for the most part with the hypothesis that some strains produce uninucleate spores, whereas others produce multinucleate spores. Conidia from *S. griseus* recombinants, for example, yielded one or the other parental type, indicating

that the spores were either uninucleate or that all nuclei in a spore had a common origin.[12] The genetic data, moreover, suggested that the spore nuclei of *S. griseus* were either haploid or homozygous. Contrariwise, at least some spores of *S. coelicolor* contained two genomes; the spores, therefore, may be binucleate, multinucleate, diploid, polyploid, or aneuploid.[13, 14, 22, 23]

Saito and Ikeda have noted that reversions in auxotrophic mutants of *S. griseoflavus* produced colonies yielding both revertant and auxotrophic conidia. These workers considered this as evidence for the bipartite state of the spore nuclei in *S. griseoflavus*. Inasmuch as only a single nucleus was observed microscopically in each spore, they concluded that the spore nuclei were diploid. The same result, however, would have been obtained with haploid, uninucleate spores if the mutation occurred in a polytenic chromosome or in chromatids. Alternatively, two haploid spore nuclei could conceivably have been appressed closely, thereby appearing as a single nucleus.

Saito and Ikeda also observed that the frequency with which mutants were recovered after ultraviolet irradiation was increased if the spores were preincubated before testing. These authors considered the delayed appearance of mutants as corroboration of their hypothesis that spores of *S. griseoflavus* contain two genomes. The effects of preincubation, accordingly, were attributed to segregation delay.[24] There are several explanations other than segregation delay that can account for the effects of preincubation. These include delayed mutation, delayed expression of the phenotype, and a lag resulting from the distribution of strands in polytenic chromosomes.[25]

Latarjet and Ephrussi have suggested that there is a single target sensitive to X rays for each chromosome set; dose-survival relationships for X-irradiated spores, therefore, can provide some information about the genetic composition of streptomycete conidia.[26] Kelner has demonstrated that the dose-survival relationship for *S. flaveolus* spores irradiated with X rays was exponential, that is, resulted in a one-hit curve.[27] The conidia of *S. griseus* and *S. kitasatoensis* have also been found to give one-hit survival curves with X rays, thereby supporting the hypothesis that the nuclei of these spores are uninucleate and haploid.[20, 21]

The excellent work of Saito and Ikeda has shown that conidia of *S. griseoflavus* gave a two-hit survival curve; these investigators have interpreted this to mean that the spores contained two sets of chromosomes. Because their cytological studies revealed only a single nucleus in each conidiospore, they concluded that *S. griseoflavus* is diploid. Saito and Ikeda considered the response of *S. coelicolor* conidia to be in the one-hit category; the data given, however, were equally consistent with a two-hit survival curve.[20]

Although radiokinetic evidence is a useful adjunct to genetic studies, the genetic composition of streptomycete conidia cannot be determined solely by this method. Extrapolations of curves plotted as the logarithm of percentage survival against X-ray dosage are greatly affected by minor variations. Moreover, exceptions to the single target per genome relationship are known; Pomper *et al.*, for example, have demonstrated that some diploid yeast yielded one-hit survival curves.[28] In addition, Puck *et al.*[29] have found that HeLa cells gave only a two-hit response, even though the chromosome number was about triple the haploid number.[30] Saito and Ikeda, nevertheless, deserve

commendation for their credible radiokinetic studies with *S. griseus*, *S. kitasatoensis*, and *S. griseoflavus*.

Genetic Composition of Vegetative Mycelia

Fusion of hyphae originating from vegetative mycelia of two differently marked mutants growing together was observed within 36 hours after the spores were plated (Bradley *et al.*, elsewhere in this monograph). Recombinant phenotypes, however, could not be isolated until the complementary, auxotrophic strains of *S. coelicolor* had been grown in combination for 60 hours.[11] Possible explanations for the inability to detect recombinants earlier include the following: (1) the two classes of parental nuclei may not be sufficiently mixed to permit the detection of a heterokaryotic spore or hyphal segment; (2) heterozygotes may arise as a result of very rare nuclear fusions; (3) recombinant nuclei may be masked by an excess of parental nuclei; or (4) recombinant phenotypes may be manifested only after sporogenesis. Inasmuch as recombinant phenotypes have been recovered directly from the vegetative mycelium, sporogenesis is not an obligatory prerequisite for recombination.[13, 23]

The three remaining alternatives are similar in that both of the parental nuclei are present within the hyphae of recombinants; additionally, these hyphae may contain nuclei formed by fusion and segregation. Clearly, genetically diverse nuclei can exist in the vicinity of one another, thereby establishing, in most instances, a functional heterokaryon. If the different nuclei fail to interact, or if the nuclei are distributed unequally, unbalanced heterokaryons may form. Nutritionally unbalanced heterokaryosis has been observed regularly in crosses between doubly deficient pairs of *S. fradiae* mutants or *S. coelicolor* mutants.[14] These unbalanced heterokaryons grew only on supplemented minimal medium, even though both of the parental genomes were found among conidia picked from each of the colonies. These nutritionally unbalanced heterokaryons were probably the same as the anomalous heterokaryons described earlier by Bradley and Lederberg.[12]

Dissociation

Recombinant colonies sporadically developed sectors having nutritional requirements different from the remainder of the colony; also, spores from recombinants have yielded rare dissociates with nutritional requirements unlike the colonies from which the inocula were prepared. Moreover, characteristics not recognized previously in either parent have arisen among colonies derived from spores; unfamiliar auxotrophic markers, for example, have been found as dissociates from recombinants. Such novel phenotypes can result from new mutations or from the unmasking of preexisting requirements by genic recombination. Although the latter hypothesis cannot be conclusively ruled out at present, it is unlikely because: (1) the same mutant phenotypes have arisen in several different strains of diverse ancestry, and (2) stable genic recombinants have not been found with respect to the input auxotrophic markers. New mutants may be induced by heterokaryotic protoplasm, as suggested by Raper and San Antonio for *Schizophyllum commune*.[31] Alternatively, the mutations may be spontaneous, but the variant nuclei may tend to remain localized, thereby making their detection more probable. Because of

subjective selection for atypical growth habits, the frequent recovery of novel phenotypes can be an artifact.[22] Moreover, the parental and nonparental dissociates that I recovered from recombinants may be the result of an interplay of fortuitous factors. Obviously, the auxotrophic stocks used bore readily obtainable markers; mutants requiring added arginine, methionine, cystine, tryptophan, uracil, or adenine, for example, were found ten times more frequently after ultraviolet irradiation than were mutants requiring valine, serine, threonine, or tyrosine. If the combined effects of subjective selection, heterokaryotic mutagenesis, and higher mutation rates for parental markers were operative, an excess of parental dissociates could have been produced.

I do not believe that the several novel and diverse dissociates found during serial subculture of recombinants are artifacts, because (1) the parental phenotypes were usually stable, (2) several types of dissociates were recovered from a single clone, and (3) the progeny sometimes differed from the preceding colony with respect to two or more characteristics. Indeed, dissociation seems to reflect some fundamental genetic or physiological process rather than an accumulation of mutations.

One of the significant difficulties encountered in the study of the genetics of streptomycetes has been the frequency of penetrance and the variability in the expressivity of a character. Several years ago, Stanier noted that the capacity to utilize agar was inherited variably in certain wild-type strains of *S. coelicolor*.[32] For example, only 5 per cent of the wild-type spores yielded colonies that produced gelase. Surprisingly, more than 90 per cent of the spores from gelase-producing colonies were unable to decompose agar. Variable expressivity may result from (1) fluctuations in the state of the gene; (2) instability of the genic content of the nuclei, thereby producing dosage effects; (3) changes in the proportions of the diverse nuclei; or (4) failure of the metabolic machinery to express the respective phenotype. Recessive characters contributed by each parent have been recognized in individual recombinants that subsequently yielded both of the respective dominant expressions (Bradley, in this monograph); accordingly, any hypothesis relying on the ratio of intact, haploid parental nuclei within a hyphal segment is untenable. Inasmuch as expressivity of unselected markers was dependent upon the particular markers selected, multiple instabilities in the state of the genes and multiple breakdowns in the physiological machinery seem unlikely. Fluctuation in genic dosage, consequently, is the preferable alternative. Variations in genic dosage can result from (1) inconstancy of the ploidy of the nuclei and (2) unequal distribution of the chromosomes to the daughter nuclei. Some cytological evidence has been presented indicating that the nuclear material of the streptomycetes is somewhat diffuse and divides amitotically.[33] Because each parental genome tended to stay together, a mechanism similar to that described in the Suctorians by Grell may exist.[34] Streptomycete nuclei, therefore, may be merely an amorphous association of several complete and partial genomes; the chromosomes, nevertheless, preferentially may form units consisting of a single genome. The unattached chromosomes may be distributed unequally during amitosis, thereby providing the mechanism for continuing variation.

The Sermontis have obtained several apparently stable phenotypes as dissociates from a single clone.[23] This can be explained by (1) amitosis with

aneuploidy or (2) repeated nuclear fusion and genic recombination. Indeed, the difficulties encountered may be the result of the manifold processes occurring simultaneously. Heterokaryosis, parasexuality, transduction, transformation, and gene conversion—conceivably all can be operative.

Resolution of the Problems

An understanding of the recombinational process(es) has been complicated because dominant markers have been used as selective characters. Classic heterokaryosis can be avoided if recessive markers are selected. I have attempted, with little success, to select for streptomycin-resistant, bacteriophage-resistant recombinants from a streptomycin-sensitive, phage-resistant parent and a streptomycin-resistant, phage-sensitive parent. Moreover, the behavior of unselected markers must be analyzed thoroughly. The auxotrophic characters used to date have been selected preferentially or adversely on complete medium and appropriately supplemented media. Consequently, quantitative data, relying upon the behavior of auxotrophic markers, has no real significance. Stable recombinants must be back-crossed to the parents and crossed with one another. The genetic composition of these organisms cannot be determined accurately in any other way.

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Discussion of the Paper

Waclaw Szybalski (*Institute of Microbiology, Rutgers, The State University, New Brunswick, N. J.*): S. G. Bradley has postulated that "different (streptomycin-resistant) genes, one dominant and the other recessive, may be involved" to explain the results of reciprocal crosses in *S. coelicolor*. This seems improbable on the basis of our findings that (1) several independently isolated streptomycin-resistant mutants of *S. coelicolor* gave the same results, and (2) all streptomycin-resistant markers involved in similar experiments with *S. fradiae* were shown to be recessive by heterokaryotic tests (see TABLES 2, 6, and 7 of our paper elsewhere in this monograph). Complete diploidy or heterokaryosis therefore could be excluded as the underlying cause for prototrophic recombinations observed with our *S. coelicolor* and *S. fradiae* strains.

Another comment pertains to the definition of heterokaryosis by Bradley. The notion of cooperation as applied by him should be limited to nutritionally balanced heterokaryosis, and the adjective genetic might be misleading. His definition might well be modified to read as follows: heterokaryosis is the presence of discrete nuclei of diverse type in a common cytoplasmic field.

D. Braendle (*Abbott Laboratories, North Chicago, Ill.*): The formation of nutritionally balanced heterokaryons is discussed by many contributors to this monograph. This term describes a heterokaryotic mycelium in which nutritionally deficient, discrete nuclei of both parental types, well intermixed and in proper proportion, contribute reciprocally required intermediates to the common cytoplasm, with resultant prototrophic growth. The strain of *S. coelicolor* described by G. Sermonti did not form stable prototrophic heterokaryotic

colonies, although there were convincing indications that fusion between strains occurred, as evidenced by the production of stable recombinants. The failure of these heterokaryons to show prototrophic growth finds possible explanation in: (1) inefficiency of biochemical cooperation between the unlike nuclei due to barriers of unknown nature; (2) inefficient mixing of the multiplying nuclei throughout the mycelium; or (3) an improper numerical ratio of the cooperating nuclei. These hypothetical defects would allow only a very transient heterokaryotic phase, long enough, however, to bring the unlike nuclei into close contact and provide opportunity for karyogamy and recombination. On the basis of our work on heterokaryotic compatibility in *S. fradiae*, it might be of considerable interest to investigate whether the frequency of heterokaryosis between such selectively marked recombinants is different from that between the parents, and if more permanent heterokaryosis could be produced between such strains. On the other hand, the failure of well-balanced, prototrophic heterokaryons to form on selective minimal media actually might facilitate the observation and isolation of true recombinants endowed with decided nutritional advantages.

It also has been observed generally that heterokaryons of *Streptomyces* are unstable, producing only parental types of conidia. In this respect, the interpretation chosen by S. G. Bradley is questionable. It seems possible that his stable heterokaryons actually might represent a rather stable diploid phase in that strain of *S. coelicolor*. The impossibility of propagating stable heterokaryons through uninuclear conidia (or conidia with a few nuclei) is pointed out by K. F. Gregory elsewhere in this monograph. We wonder whether Bradley has performed any crossing experiments with his strains, reciprocally marked with either streptomycin or phage resistance. Are the stable prototrophic progenies of the cross $a^-b^-c^+d^+S^R \times a^+b^+c^-d^-S^s$ and of the cross $a^-b^-c^+d^+S^s \times a^+b^+c^-d^-S^R$ uniformly streptomycin-sensitive? This was not our experience in similar reciprocal crosses with *S. coelicolor* and *S. fradiae*, and this was one of the reasons why the interpretation of stable diploidy was excluded, as discussed in our main paper elsewhere in this monograph. The recessiveness of the many streptomycin-resistant markers used in our experiments was confirmed in separate studies, in which heterokaryons between the sensitive and resistant strains were synthesized.

BRADLEY: As I have pointed out earlier, reciprocal crosses are essential prerequisites for an understanding of the genetic process(es) in the streptomycetes. Before a reciprocal cross is valid, the genes in question must be proved to be alleles and the gene pairs controlling each expression must be identical. Unfortunately, Szybalski and Braendle did not report the results of the $S_1^R \times S_2^R$ cross; therefore, it has not been established that these two genes are identical alleles. Moreover, it must be noted that the expressivity of the genes controlling streptomycin susceptibility is influenced by the selective medium.

RADIATION AS A CYTOGENETIC TOOL*

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The application of cytogenetic principles to bacteria and actinomycetes has proved to be a difficult task that has met with varying success. This is due in part to the fact that many of the useful classic cytological and cytogenetic procedures as yet cannot be applied directly to these organisms. Size of the cells alone makes cytological studies difficult, although significant advances in the understanding of the cytology of microorganisms have been made in recent years. However, despite the excellent work done on nuclear cytology by DeLamater (1954), there is still no good evidence that an actual, classic cytological mitotic division occurs. As pointed out by Robinow (1956), other mechanisms of nuclear division are equally feasible in light of our present knowledge. There is little doubt that a genetic mitotic division occurs, however.

In making a thorough study of the actinomycetes, it is necessary to determine and understand the life cycle. A survey of literature on the life cycles of actinomycetes and bacteria reveals differences of opinion as to what constitutes a valid life cycle. In work in my laboratory, I have considered a life cycle to be the series of morphologic and cytological changes that occur in the development of the culture from youth to old age. These changes must include a demonstrable change in ploidy. Since direct chromosome counts in bacteria and actinomycetes are of questionable validity, it is necessary to employ a different experimental tool to detect these ploidy changes. Despite their many difficulties, radiation studies appear to be most useful as an adjunct to direct cytological observation.

The use of radiation in nuclear studies can be subject to criticism, since radiation biologists are not in agreement as to the primary effect of radiation on the cell (Zelle and Hollaender, 1955). Although there is much evidence for a nuclear site of action (presumably genetic), there also exists evidence for a cytoplasmic site. In all probability both nuclear and cytoplasmic damage occur, and the only valid use of radiation as a cytogenetic tool would be under conditions in which primarily nuclear or genetic damage was detected.

The use of radiation procedures in a study of *Nocardia corallina* has served to test the application of these procedures to the study of other actinomycetes. Techniques proved to be of value with *Nocardia* should be of equal value when applied to *Streptomyces* or other actinomycetes. *N. corallina* was chosen for these studies since it possessed a relatively simple apparent life cycle (Webb *et al.*, 1954). The existence of a unicellular, uninuclear coccoidal stage in the growth cycle made the organism adaptable to accurate radiation studies.

The kinetics of irradiation inactivation of a culture of microorganisms can give an indication of the average number of hits or events required to inactivate the biological unit. This applies only to ionizing irradiations since the dose-

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effect picture is much more complex when ultraviolet light is used as the source of irradiation. Such kinetic study gives no indication of the nature of the biological unit involved. This unit may be cell clumps, polycellular units, multinuclear cells, or uninucleate cells. Obviously, if any interpretations on ploidy are to be made on the basis of inactivation kinetics, it is necessary to accompany the radiation studies with careful cytological and morphologic studies. If a uninuclear, unicellular, single-cell suspension is inactivated at an exponential rate, it is an indication that only a single event is required for inactivation, and it can be assumed that the culture is probably haploid. Such assumption is based on the theory that the primary radiation effect detected is nuclear. If a sigmoid or multievent inactivation response is obtained on such a culture, it can be assumed that the culture is primarily diploid. Atwood and Norman (1949) have discussed the interpretation of multihit inactivation curves, and Tobias (1952) has developed a useful formulation of ploidy effects on such a curve. However, such assumptions should still be accepted as only working hypotheses. It is also possible that an apparent diploid population of a microorganism is actually a heterogenous population of mixed ploidy and should be referred to as mixoploid (Clark and Webb, 1955).

A better indication of a mixoploid or diploid population can be obtained by application of the unpaired defect theory of Tobias (1952). This theory has been successfully used in a study on *N. corallina* (Clark and Webb, 1957) and helped establish the life cycle in this organism (Webb and Clark, 1957). This theory is based on the inheritable nature of radiation damage that leads to cellular inactivation. In a diploid cell, such defects (which are analogous to recessive lethals) may be unpaired and, as a result, the cell remains viable. Pairing of these defects on subsequent irradiation leads to inactivation. No such pairing would be observable in a haploid cell. The results of a typical experiment are shown in FIGURE 1. In this experiment the X-ray inactivation curve of the parent culture is indicated by Curve 1. Colonies arising from cells that survived an X-irradiation dose sufficient to give only 25 per cent inactivation were pooled and the resulting culture again irradiated. This was repeated several times; Curve 2 shows the results obtained from the fourth successive irradiation. The resulting sensitivity is contrary to what would be expected from the selective action of the radiation, and the change from a sigmoidal to an exponential response is compatible with the unpaired defect theory. Full details of these experiments have been published previously (Clark and Webb, 1957).

Additional successive irradiation of *N. corallina* cultures resulted in selection for the resistant cells present in the parent population. The inactivation curve remained exponential, indicating a continued saturation of unpaired defects in the surviving cells. These cells are present in few numbers in the parent population, but they have been isolated and found to give a sigmoidal survivor response on primary irradiation (Clark and Frady, 1959). The existence of these cells and their behavior on successive irradiation offers additional confirmation of the validity of the unpaired defect theory. It is realized that complete confirmation of this theory has not yet been obtained; in fact, some discrepancies have been found in yeast cells. Cytoplasmic inheritance could be used in an explanation of the results obtained, but nuclear inheritance ap-

pears to be the most probable explanation on the basis of present knowledge. It appears that the use of successive irradiation, accompanied by adequate cytological studies and, where possible, genetic analysis, affords the best present method of determining the ploidy of a population of microorganisms.

The study of radiation inactivation of multinuclear units yields less clear results and more difficult interpretations. If comparative studies are made be-

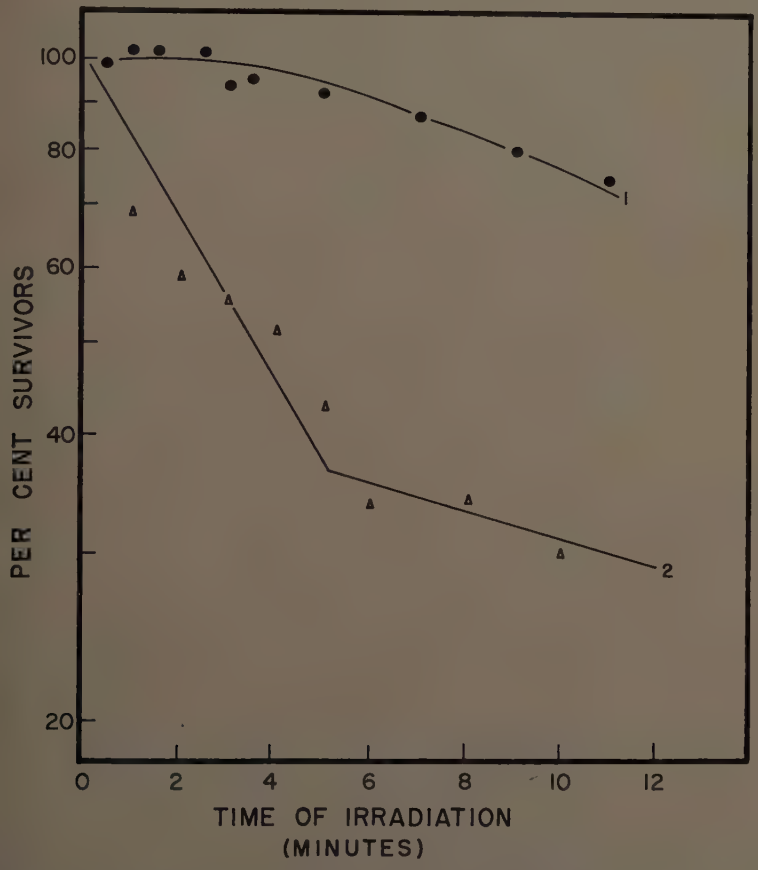

FIGURE 1. Effect of successive X irradiation on *Nocardia corallina*. (1) Survivor response of parent culture; (2) survivor response after the fourth successive X irradiation.

tween uninuclear and multinuclear units of the same culture, however, useful information can be obtained. This is illustrated by the work of Webb and Clark (1957). In *N. corallina*, the primary life cycle involves uninucleate coccoidal cells that germinate when transferred to a fresh medium and develop into coenocytic hyphae. After maximum growth, depending on cultural conditions, the hyphae fragment by cross-septum formation into binucleate bacillary cells. The bacillary nuclei fuse and form the uninucleate coccoidal cell that terminates the primary life cycle. Successive irradiation experiments showed the coccoidal cells to be diploid or mixoploid. However, if this were a true

life cycle, the question would remain as to which phase involved ploidy change. Cytological studies suggested that the coccoidal germination involved a nuclear reduction division. If such were the case, the hyphae would be expected to be more radiation-sensitive than the coccoid, even though the hyphae were multinucleate. A multinucleate, haploid cell would not require the paired events necessary to inactivate the coccoid and, even though a multievent inactivation response would be expected, the dose-rate inactivation should be less. As shown in TABLE 1, this was not found to be true. The hyphae were found to be more resistant than the coccoids, and the variation in resistance of the hyphae at various cultural ages was correlated with the numbers of nuclei per hyphal unit. The bacillary cells yielded essentially a two-event inactivation response indicating that these cells contained two haploid cells. It was thus concluded that the reduction division occurred on fragmentation and not on germination as originally thought. Without comparative studies, the dose-effect study on the multinucleate hyphae would have had little meaning.

TABLE 1

X-IRRADIATION SURVIVAL RESPONSES OF CELLS FROM VARIOUS GROWTH CYCLE STAGES OF *NOCARDIA CORALLINA*

Stage of growth	Percentages of survivors at doses of			
	12 kr	24 kr	30 kr	42 kr
7-day coccoids	95	55	53	27
3¼-hour germinating coccoids	96	70	60	38
7¼-hour hyphae	98	97	90	70
25½-hour bacillary cell	45	8	2	—

Another use of radiation response is as a genetic marker. Repeated attempts to obtain adequate genetic markers in *N. corallina* cultures have not been successful. Drug resistance or biochemical deficiencies were found to be too unstable for experimental work. Colony morphology and pigment mutants were isolated, which allowed the demonstration of gene recombination (Webb, 1956), but these were unsuitable for other work. Cells of varying resistance to irradiation were isolated from the parent culture (Clark and Frady, 1959). These included cultures of cells very resistant to irradiation, as well as those very sensitive to irradiation. The response of the sensitive cultures was found to be due to the presence of an unusually high percentage of bacillary cells. However, the resistant cultures apparently were attributable to a recessive allele. On repeated subculture, these cultures were found to revert to the parent type. Each subculture allowed one life cycle and, since each life cycle involves heterokaryon formation as well as gene recombination (Webb, 1956), the radiation-resistance character apparently was diluted to the point of phenotypic extinction. To test the validity of such assumption, a culture that had been irradiated successively four times was studied for reversion. This culture contained many unpaired defects, and pairing of such defects through heterokaryon formation would be expected to result in their eventual loss from the culture. The results of a typical experiment are given in FIGURE 2. This

reversion not only gave information on the heterokaryons, but also yielded additional evidence that the primary site of radiation damage, as detected under the experimental conditions used, was nuclear.

On the basis of this work, it appears that there are at least three ways in which radiation studies can be incorporated into a cytogenetic study of the actinomycetes.

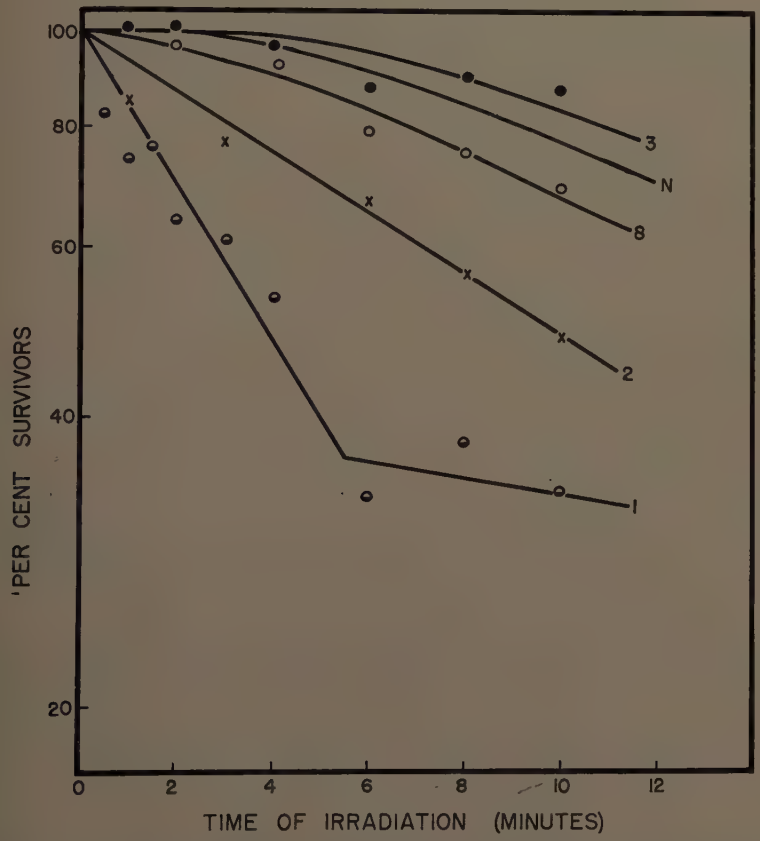

FIGURE 2. X-irradiation dose-survival response of previously X-irradiated *Nocardia corallina* coccoids upon transfer without additional radiation treatment. (1) Fourth successive X-irradiation (before transfer); (2) survival curve after one transfer; (3) survival curve after two transfers; (8) survival curve after seven transfers; and (N) X-irradiation dose-survival curve of parent culture.

(1) The survival response to X irradiation correlated with cytological studies can give an indication of the genetic nature of the nucleus of the majority of cells in a culture. In such work, to avoid false interpretations it is necessary to determine the nature of the biological unit that is inactivated. Comparison of dose-effect responses of multinucleate units to those of uninucleate cells of predetermined ploidy can yield information on the ploidy of the nuclei in the multinucleate cell.

(2) Successive irradiation of cultures, in which cells surviving a predeter-

mined dose of irradiation are pooled for each subsequent irradiation, is the preferred method for differentiating between haploid and diploid or mixoploid cultures. If each subsequent irradiation shows increased sensitivity to irradiation, it can be assumed that the cells are diploid or mixoploid. A haploid culture will have a uniform survival response or show increased resistance due to the selection of the irradiation for radiation-resistant forms. Since such resistant forms may also be present in a mixoploid population, it is necessary to choose an irradiation dose sufficiently low to assure detection of cells containing unpaired defects. A high irradiation dose can cause complete defect saturation in a mixoploid population, so that subsequent irradiations would yield increased resistance due to selection. Such a pitfall must be avoided carefully in successive irradiation experiments.

(3) Irradiation resistance can be used as a genetic marker. Although other genetic markers would be superior, irradiation resistance can be used to advantage when other markers are not available.

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Part II. Genetics of Antibiotic Production and Studies on Lysogeny in *Streptomyces*

INTRODUCTORY REMARKS

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For many years mutation and selection have been used effectively in the isolation of strains of fungi and *Streptomyces*; this has resulted in improved and higher antibiotic production. Although the use of genetic techniques for breeding and recombination has been a theoretically promising possibility in this area, it has been receiving increased attention only relatively recently. The papers presented in this monograph represent definite progress towards the fulfillment of exciting prospects inherent in the application of genetic methods to our understanding of the roles of genetic factors in antibiotic biosynthesis, to the quantitative improvement of antibiotic production, and to the production of newer and more desirable antibiotics.

That the time is now ripe for an all-out attack on the genetics of antibiotic-producing microorganisms is evidenced by the number of investigators and laboratories now working in this area. It seems especially appropriate that this field is represented by papers in this monograph by investigators from many parts of the world: Canada, Belgium, England, Italy, Japan, Russia, as well as the United States.

It is to be hoped and expected that this type of international effort and cooperation will lead to the increasingly effective application of genetic concepts and techniques to the problems of antibiotic production, and to significant improvements in human health and freedom from disease.

GENETICS OF ORGANISMS PRODUCING TETRACYCLINES

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Some Regularities in Induced Variation and Selection of Tetracycline-Producing Actinomycetes

It is well known that the use of mutagenic factors has played an important role in breeding highly active antibiotic-producing strains. The most significant successes have been achieved in the selection of the penicillin-producing organism *Penicillium chrysogenum* and the streptomycin-producing organism *Streptomyces griseus*, the activity of which has been increased many times. The problems of selection among other antibiotic producers, particularly among the chlortetracycline-producing organism *S. aureofaciens* and the oxytetracycline-producing organism *S. rimosus* seldom are described in the literature. In one series of papers[1-3] there are some indications of obtaining highly productive mutants of the above-mentioned producers as a result of application of ultraviolet light and nitrogen mustard. More detailed data on the variation and selection of *S. aureofaciens* have been presented by Katagiiri.[4] Data on the selection of *S. rimosus* have been published by Borenstajn and Wolf.[5]

However, all these studies are primarily of an empirical character. They contain no data concerning the methods of the most effective treatment with different mutagenic factors. Little attention is paid to the regularities of the induced variation; in particular, to the variation with respect to antibiotic production. The absence of precise comparative data on the activity of newly obtained and initial strains also may be considered shortcomings of these studies.

For several years the selection work in our laboratory has been conducted with a number of antibiotic producers, including *S. aureofaciens* and *S. rimosus*.

To increase the variation of *S. rimosus* only ultraviolet irradiation was used, whereas in the experiments with *S. aureofaciens*, in addition to ultraviolet irradiation, treatment with X rays and ethyleneimine was used. These factors were applied both separately and in combinations. In the course of this work some interesting facts and regularities were observed; these are believed to be worthy of attention and will be described later.

As a result of this work, highly active strains of both producers were obtained.

Materials and Methods

As the starting material, strain No. 77 of *S. aureofaciens* and strains No. 101 and No. 8229 of *S. rimosus* were used. In the course of the selection, strains LS-536, No. 112 and LS-B16 were obtained from strain No. 77 of *S. aureofaciens* and strains LS-T118 and LS-T293 from strain 8229 of *S. rimosus*. All these strains were used at various stages of the experiments.

As a source of ultraviolet light, bactericidal ultraviolet lamp BUV 30, emitting chiefly the rays at wave length 2537Å, was used. The doses of ultraviolet

light were measured by dosimeter UVM-5. As a source of roentgen rays, a short-focus tube of roentgen apparatus RUM-7 rated at 60 kv. and 20 mAmp. was used.

Suspensions of aerial spores of *S. aureofaciens* and *S. rimosus*, chiefly containing single spores, were subjected to irradiations. The cultures were grown on agar corn-steep media. The fermentations were carried out in fluid corn-steep media in shake flasks.

The irradiation technique and the cultivation procedures, as well as the medium compositions and the cultivation conditions, are described in our previous papers.[1, 6]

Combined Effect of Mutagenic Factors on S. aureofaciens

It is known that in some cases the combined treatment with mutagenic factors produces a synergistic effect.

In this respect the data described by Swanson *et al.*[7, 8] are the most significant ones. These investigators found that pretreatment of *Aspergillus terreus* conidia with low, nonmutagenic doses of nitrogen mustard greatly increases the mutagenic effect of ultraviolet irradiation.

As far as the combined effect of X rays and ultraviolet rays is concerned, the data are contradictory. Some authors report that these factors produce a synergistic effect;[7] others, on the contrary, point out that ultraviolet irradiation lowers the effectiveness of the subsequent irradiation with X rays.[9]

In our search for the most effective treatment with mutagenic factors, we have studied the combined effect of ethyleneimine and ultraviolet light, on the one hand, and that of ultraviolet and X rays, on the other.

Combined Effect of Ethyleneimine and Ultraviolet Light

The experiments were carried out with *S. aureofaciens* strain No. 112 for the purpose of studying lethal and mutagenic effects of ethyleneimine and ultraviolet light applied separately and in combination.[10] In the latter case the spores of *S. aureofaciens* were immersed in ethyleneimine solutions of different concentrations (1:5000; 1:6000; 1:7000) for 20 hours and then irradiated with increasing doses of ultraviolet light, or the spore suspensions were first irradiated with ultraviolet light and then immersed in ethyleneimine solutions. The data on the survival of spores treated in different ways with the mutagenic factors are presented in TABLE 1.

From this table it is evident that the succession of treatment with these factors is of great importance. It was found that the lethal effect was much higher when the spores were first treated with ethyleneimine and then irradiated with ultraviolet light (ethyleneimine → UV light), than in the case of the reverse succession of the treatment with these factors (UV light → ethyleneimine).

The preliminary treatment with ethyleneimine increased not only the lethal effect of ultraviolet rays, but also its mutagenic effect. In this case a considerable increase in the frequency of mutations induced by ultraviolet rays was observed even at low doses of ethyleneimine (1:7000); this did not affect the viability of spores and did not produce a marked increase in the mutation

frequency. The corresponding data on the frequency of morphologic mutations in *S. aureofaciens* strain 112 are presented in FIGURE 1.

From this figure it is evident that the frequency of mutations in spores preliminarily treated with ethylenimine increases continuously with the increase of ultraviolet doses. Even at the dose of 10,000 ergs/sq. mm. the muta-

TABLE 1

SURVIVAL OF SPORES OF *S. AUREOFACIENS* STRAIN NO. 112 AFTER SEPARATE AND COMBINED TREATMENT WITH ETHYLENEIMINE (EI) AND ULTRAVIOLET LIGHT (UV)

UV dose ergs/sq. mm.	Survival						
	UV	EI (1:5000, 20 hours)			EI (1:6000, 20 hours)		
		Without UV	UV → EI	EI → UV	Without UV	UV → EI	EI → UV
2000	1.9×10^{-3}	1.5×10^{-3}	5×10^{-4}	2×10^{-6}	8×10^{-3}	6.3×10^{-3}	5×10^{-6}
4000	8×10^{-4}	1.5×10^{-3}	4×10^{-4}	1×10^{-6}	8×10^{-3}	4×10^{-4}	2×10^{-6}
6000	3×10^{-4}	1.5×10^{-3}	6×10^{-5}	8×10^{-7}	8×10^{-3}	2.7×10^{-4}	7×10^{-7}
10000	4×10^{-5}	1.5×10^{-3}	6×10^{-6}	5×10^{-7}	8×10^{-3}	1.9×10^{-5}	13×10^{-7}

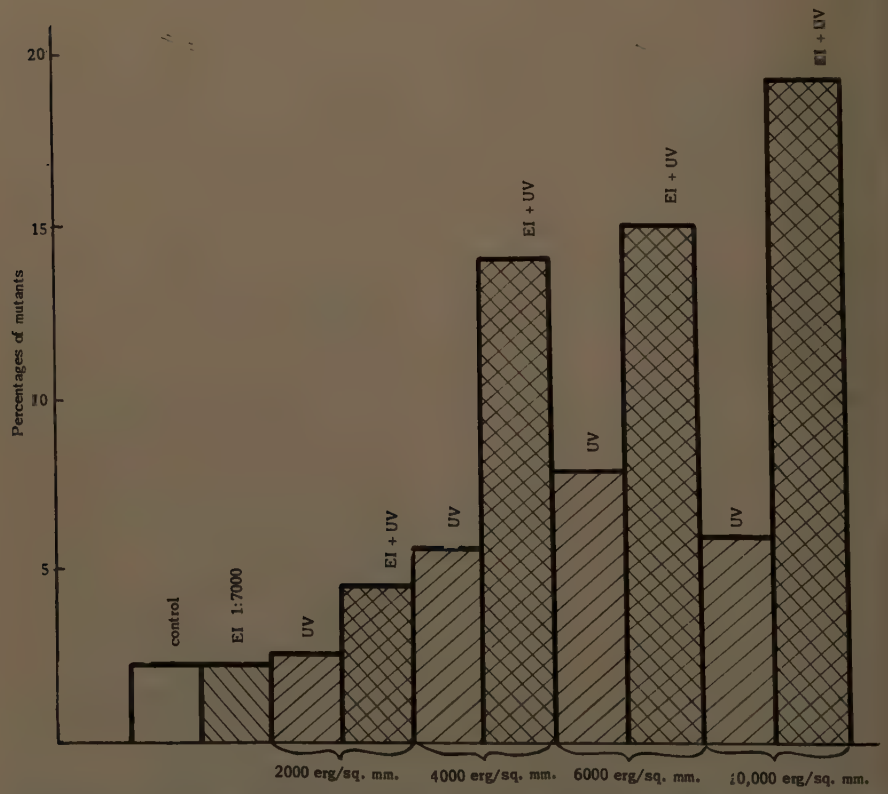

FIGURE 1. The frequency of morphologic mutations in *S. aureofaciens* strain 112 at separate and combined treatment with ethyleneimine and ultraviolet light.

tion frequency exhibits no tendency to decrease. However, when spores not treated with ethyleneimine are irradiated with ultraviolet light the frequency of mutations reaches its peak at 6000 ergs/sq. mm. and decreases with further increases of the dose. Therefore, the combined treatment with ethyleneimine and ultraviolet light results in a considerable increase of the maximum mutation frequency: the fact used by us for the selection of highly active variants of *S. aureofaciens*.

Combined Effect of Ultraviolet and X Rays

Spores of *S. aureofaciens* strain LS-B16 were used for the irradiation. In all experiments constant doses of ultraviolet and X rays were used, 4000 ergs/sq. mm. for ultraviolet light and 36,000 r for X rays at 38,520 r/min. The irradiation was performed in four ways: irradiation with ultraviolet light; irradiation with X rays; combined irradiation in the successive UV → X rays; and the combined irradiation in the succession X rays → UV. In both cases

TABLE 2

EFFECT OF COMBINED AND SEPARATE TREATMENT WITH UV AND X RAYS ON THE SURVIVAL OF SPORES OF *S. AUREOFACIENS* STRAIN LS-B16 (AVERAGE VALUES FROM 5 EXPERIMENTS)

Survival										
UV → X rays				UV rays	X rays	X rays → UV				Survival expected in case of an additive effect
Intervals between the irradiations (hours)						Intervals between the irradiations (hours)				
0	1	2	3			0	1	2	3	
2.2×10^{-5}	3.3×10^{-5}	2.5×10^{-5}	3.4×10^{-6}	3.7×10^{-5}	1.4×10^{-3}	3.5×10^{-6}	3.9×10^{-6}	9.0×10^{-6}	1.3×10^{-5}	5.5×10^{-8}

of the combined treatment the interval between irradiations was 0, 1, 2, or 3 hours. During the intervals between irradiations the suspensions were kept at 5° C. The data on the effect of the irradiations upon the viability of *S. aureofaciens* spores are presented in TABLE 2. The table shows that the lethal effect of the combined treatment with both types of radiation in any succession was much lower than that expected from their cumulative effect.

In this case, the succession of the irradiations was also of great importance. When the succession UV → X rays was used without any interval or with a one- or two-hour interval between irradiations, the viability was much higher than in the case of the reverse succession. Thus, ultraviolet light reduced the lethal effect of the subsequent X-ray irradiation.

If the interval between the irradiations was three hours, the result was quite different and the viability of the irradiated spores markedly decreased. From this fact it is possible to assume that the effect of ultraviolet irradiation is retarded.

The irradiation of the spores in the succession X rays → UV produced opposite results. In this case the increase of the interval between irradiations resulted in reduction of the lethal effect.

TABLE 3 presents the data on the effect of separate and combined irradiations with X and UV rays upon the mutation frequency of the survivors, the morphologically changed variants being considered as mutants. As is evident from this table, the mutation frequency increases markedly when the succession X rays → UV is used. When the succession is the reverse, the mutation frequency is the same as in the case of irradiation with one factor, and increases if a three-hour interval between the irradiations is used. On the contrary, when the spores are irradiated in the reverse succession, that is, in the succession X rays → UV, the mutation frequency with the use of a three-hour interval between the irradiations appears to be the lowest for this succession.

The results obtained should be considered as preliminary ones. However, even now the need for a more detailed study of the combined effect of UV and X irradiations on the mutation process in actinomycetes is obvious.

TABLE 3

COMPARATIVE MUTATION FREQUENCY WITH THE COMBINED AND SEPARATE TREATMENT OF SPORES OF *S. AUREOFACIENS* STRAIN LS-B16 BY UV AND X RAYS

Mutations (percentages)									
UV → X rays				UV rays	X rays	X rays → UV			
Intervals between the irradiations (hours)						Intervals between the irradiations (hours)			
0	1	2	3			0	1	2	3
1.77	0.72	0.6	7.45	0.42	0.1	14.2	10.77	17.2	7.62

Comparative Sensitivity of S. rimosus Strains to Ultraviolet Irradiation

The aerial spores of 4 *S. rimosus* strains, strains 101, 8229, LS-T118, and LS-T293, were irradiated with various doses of ultraviolet light ranging from 250 to 4000 ergs/sq. mm. Two to 4 experiments were carried out with every strain, and the average values obtained were used for plotting ultraviolet dose-response curves. The corresponding curves for the above-mentioned 4 strains of *S. rimosus* are presented in FIGURE 2. As is evident from FIGURE 2 the dose-response curves for the 4 *S. rimosus* strains are sigmoidal. FIGURE 2 also shows the different sensitivities of various strains to ultraviolet irradiation. The least active strain 101 appeared to be the least sensitive, while the more active strains 8229 and LS-T118 proved to be more sensitive, the most active strain LS-T118 being the most sensitive. The fourth strain LS-T293, which was also a highly active one but differed from the other strains by some physiological properties (see below), was intermediate in its sensitivity to strains 8229 and 118. Thus, on the whole, the sensitivity of *S. rimosus* strains to ultraviolet irradiation increases with the rise of their activity.

Some Peculiarities of Induced Morphologic Variation in S. rimosus

S. rimosus is characterized by its great morphologic variation, displayed especially clearly under the treatment with mutagenic factors. When treated

with ultraviolet rays, different strains of *S. rimosus* often produced the so-called mosaic or sectorial colonies, together with other types of morphologic variants. As sometimes the number of such colonies reached 20 per cent of all induced morphologic variants, it was of interest to study their peculiarities. Therefore, colonies of strains No. 101 and LS-T118 were taken and their segregation in several successive generations was studied. Spores of mycelium fragments of initial sectorial colonies were plated on an agarized corn-steep liquor medium. The colonies grown were examined and the number of colonies of different types was counted. Isolated colonies of every type (1 to 2 colonies) then were plated again. This procedure was repeated several times.

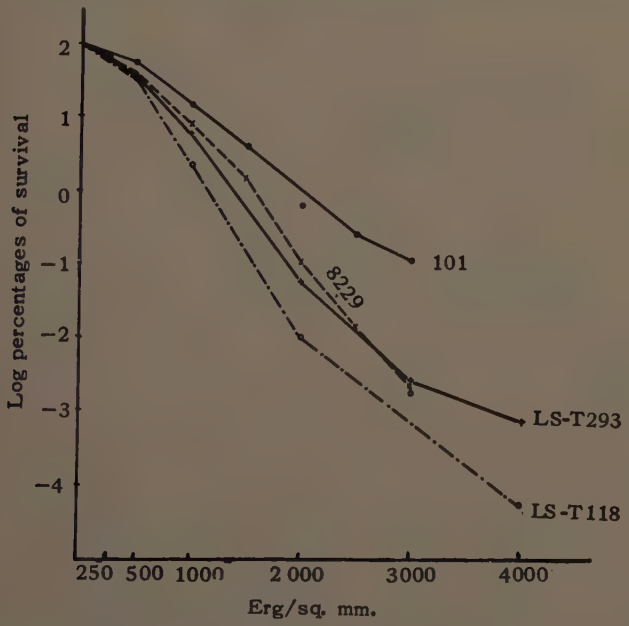

FIGURE 2. Comparative sensitivity of *S. rimosus* strains to ultraviolet irradiation.

Upon analyzing the results obtained, it was found that *S. rimosus* had two groups of variants characterized by their instability. The difference between these two variants lies in the character of their segregation.

Unstable variants of Group I (three variants were studied thoroughly) regularly segregated out two types of colonies, namely, colonies with white aerial mycelium (white colonies) and asporogenic colonies with dark brown, almost black substrate mycelium (black colonies). On being plated, the white colonies also produced white colonies. The plating of black colonies produced black colonies, as well as white, in the course of many successive generations. This is presented graphically in FIGURE 3. TABLE 4 gives data on the segregation of black colonies of two unstable variants belonging to Group I. It is evident from this table that variant M-32 is characterized by relatively low frequency of white-colony segregation. Almost all colonies obtained from variant M-409 were white, even when black colonies were plated. Both variants had the same

segregation frequency with respect to white colonies in the course of several successive generations.

The variants of the type described were found only in strain No. 101. Strain LS-T118 did not produce these variants.

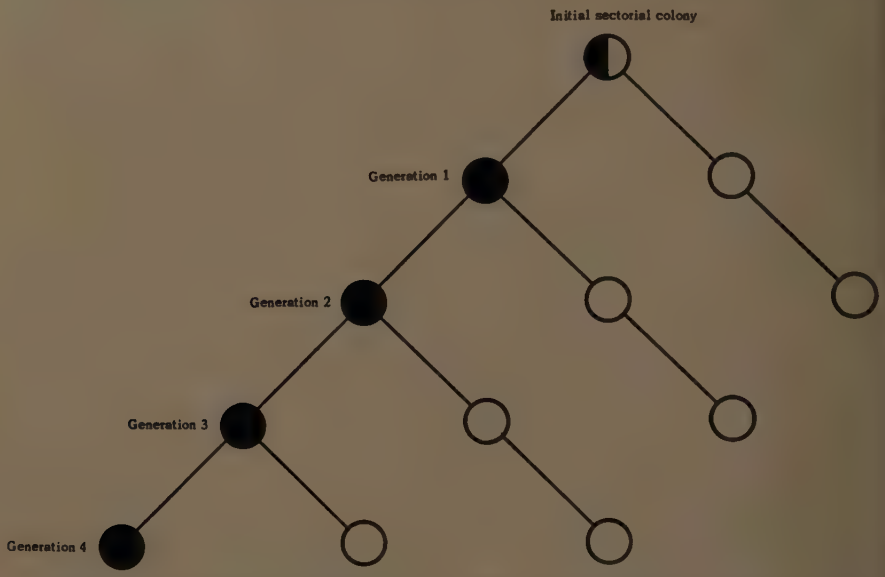

FIGURE 3. The segregation scheme of Group I unstable variants in *S. rimosus*. Key: ○, colonies with white aerial mycelium; ●, asporogenic colonies with black substrate mycelium.

TABLE 4
SEGREGATION FREQUENCY OF WHITE COLONIES IN TWO UNSTABLE VARIANTS OF GROUP I OF *S. RIMOSUS* STRAIN 101

Generation	M-32			M-409		
	Total number of colonies	White colonies and colonies with white sectors		Total number of colonies	White colonies and colonies with white sectors	
		Number	Percentages		Number	Percentages
Second	180	20	11.1 ± 2.34	307	300	97.7 ± 0.85
Third	600	96	16.0 ± 1.49	290	272	93.8 ± 1.41
Fourth	222	23	10.4 ± 2.04	426	403	94.5 ± 1.10

The segregation of unstable variants of Group II was more complicated. Both strains had these unstable variants (3 variants of strain No. 101 and 3 variants of strain No. 118 were studied thoroughly). Five to 6 or more types of colonies grew up when the spores of the initial sectorial colonies were plated. These types differed from each other in size, form, structure, color of substrate mycelium, and degree of sporulation. In their turn, colonies of almost all types segregated out several different forms. This segregation was observed in later generations. In contradistinction to unstable variants of strain No.

101, unstable variants of strain LS-T118 segregated out colonies similar to the typical colonies of this strain.

The character of segregation of unstable variants of Group II is presented graphically in FIGURE 4. TABLE 5 gives data on segregation in three types of colonies of unstable variant No. 326, strain LS-T118.

From FIGURE 4 and TABLE 5 it is evident that colonies of every type usually formed not only the same type of colonies, but some other types. Moreover, in most cases colonies of the initial type prevail.

In the process of segregation, unstable variants of Group II sometimes formed absolutely asporogenic, much folded colonies of an irregular form with soft con-

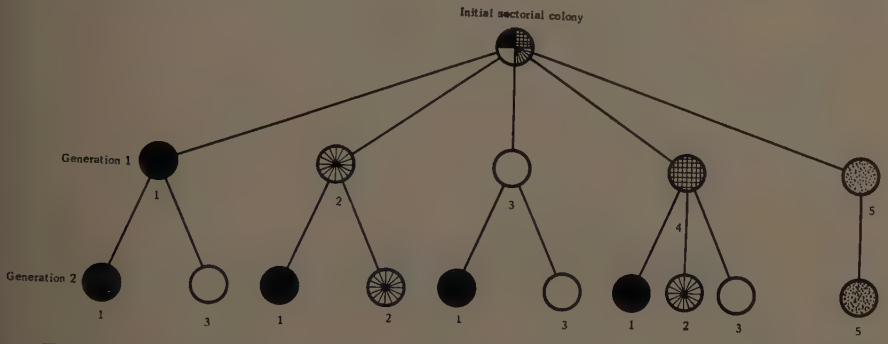

FIGURE 4. The segregation scheme of Group II unstable variants in *S. rimosus*. Numbers 1, 2, 3, 4, and 5 represent different types of colonies.

TABLE 5

SEGREGATION OF AN UNSTABLE VARIANT OF GROUP II (VARIANT NO. 326) OF *S. RIMOSUS* STRAIN LS-T118 (TOTAL VALUES FROM THREE GENERATIONS)

Colony type	Colony frequency (percentages)			
	Type I	Type II	Type IV	Type V
I	90.0	8.9	0.6	—
II	28.2	65.5	4.8	0.12
IV	65.2	31.9	0.9	0.26

sistency. These colonies consisted of short, slightly branching filaments, which are characteristic of the mycelium of proactinomycetes. These colonies produced practically no antibiotic.

As to the antibiotic activity of other types of colonies obtained from unstable variants: these produced almost the same amount of the antibiotic as the initial strain.

It is necessary to note that unstable variants appear sometimes as a result of the natural variation of *S. rimosus* strains, although this phenomenon seldom is observed.

On examination of 22,640 colonies grown from nonirradiated spores of strain No. 101, 35 sectorial colonies were found (0.15 per cent).

The data presented show that *S. rimosus* produces unstable variants of a

type described by Newcombe in *Streptomyces* T-12 (variants of Group II).[11] In addition to this type, formation of unstable variants of another type (Group I) is observed. This type is not yet described in the literature.

Variation with Respect to Antibiotic Production by S. rimosus and its Dependence upon the Dose of Ultraviolet Light

The ultraviolet irradiation of all the strains of *S. rimosus* resulted in significant variation with respect to antibiotic production. Among the variants obtained after ultraviolet irradiation there were more productive and less productive variants as compared with the initial strain, as well as absolutely inactive ones. Some variants did not differ by their productive capacity from the initial strain. The ultraviolet-induced variation with respect to the antibiotic-producing capacity was studied in detail on strain LS-T293.

The effects of 5 varied doses of ultraviolet light ranging from 500 to 4000 ergs/sq. mm. were compared. Two hundred variants were selected for examination after irradiation with each dose. The same number of variants was taken from nonirradiated culture for the comparative study of natural and induced variations. The tests for the activity of all the variants were carried out only once, with no parellel runs. Since the activity level varied from experiment to experiment, the activity of each variant was expressed as a percentage of the activity of the initial strain LS-T293 used as a control.

All the variants were classified according to their activity by 10 per cent increments of the latter, and the number of the variants in each class was counted. The data obtained are presented on FIGURE 5. Examination of the data shows that the activity of the natural variants ranges within 80 and 150 per cent of the initial strain activity, excluding the variant with an activity of about 25 per cent of the control. The variance of the activity observed in the variants induced by ultraviolet irradiation is much greater (from 0 to 170 per cent) and is shifted chiefly in the direction of low activity. At all doses of ultraviolet light, inactive or zero variants are formed (the first column on the left), and only at the lowest doses tested, that is, at 500 ergs/sq. mm., are the variants with activity exceeding that of the most active variants from nonirradiated culture (plus variants) obtained.

The frequency of active and inactive variants as dependent upon the dose applied is presented more clearly in FIGURE 6. In this case the variants with activity higher than 140 per cent of the control were considered as active ones. The dependence of the morphologic variant frequency in this experiment upon the dose used is presented graphically in the same figure. As is evident from FIGURE 6, the frequency of all the variant types at first increases with the increase of the ultraviolet dose and then decreases after reaching a certain level. In addition, the frequency of inactive variants reaches its maximum at the same dose as that of the morphologic variants, while the frequency of plus variants reaches its maximum much earlier.

Further analyses of the ultraviolet-induced variation showed that the coincidence in the formation of inactive and morphologic variants is not casual. Most morphologic variants were found to be characterized by lower activity, whereas variants with higher morphologic activity did not differ more often from the initial strain. Data on the activity of morphologically changed and

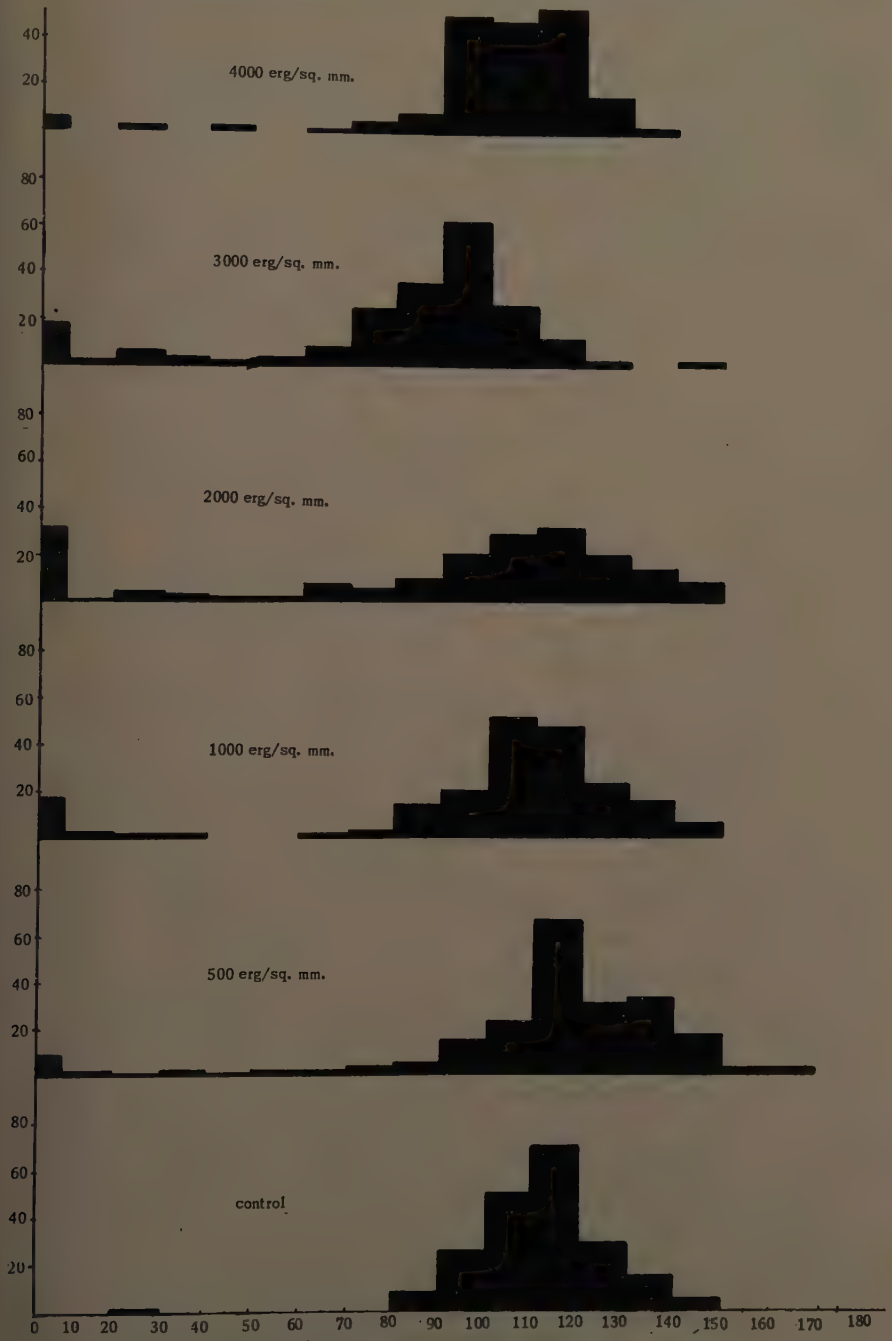

FIGURE 5. The range of variation with respect to antibiotic production in *S. rimosus* strain LS-T293 as dependent upon the dose of ultraviolet light.

unchanged variants, for all the doses of ultraviolet light, are presented in FIGURE 7. These data convincingly demonstrate that all the variants with zero activity, as well as most variants with lower activity, differ from the initial strain by their morphology. By contrast, most variants with higher morphologic activity are similar to the initial strain. Therefore, the fact that an increase in the frequency of morphologic variants is accompanied by an increase in the frequency of inactive variants and vice versa is quite natural.

The material presented allows the conclusion to be drawn that the frequency of plus and minus variants in *S. rimosus* depends upon the dose of ultraviolet light. Moreover, the maximum frequency of active variants is reached at lower doses than that of inactive and morphologic ones. It should be noted

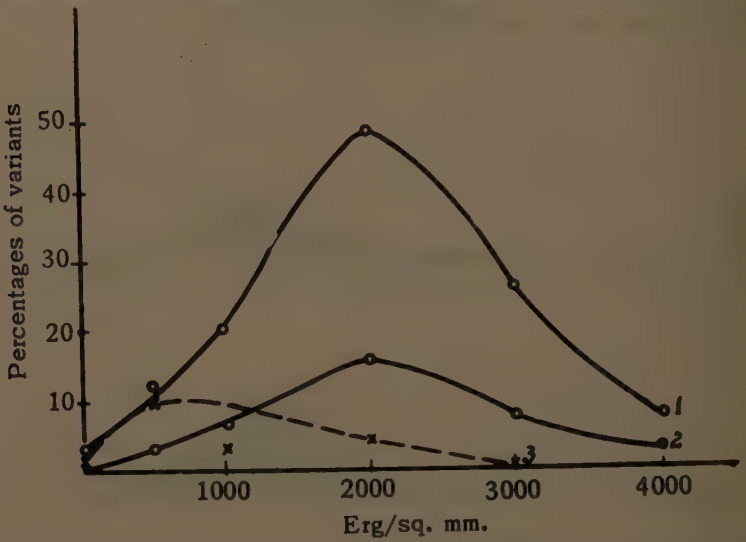

FIGURE 6. The frequency of active, inactive, and morphologic variants in *S. rimosus* strain LS-T293 as dependent upon the dose of ultraviolet light. Curve 1 represents morphologic variants; curve 2, inactive variants; and curve 3, active variants.

that the same conclusions were drawn on the basis of studying the variation with respect to antibiotic-producing property in other antibiotic producers of actinomycete nature.[12]

Results of Selection Among S. rimosus and S. aureofaciens

As a result of multistage selection among active variants obtained after the application of various mutagenic factors, new tetracycline-producing strains, considerably exceeding by their activity the initial forms, were introduced.

The selection of the active variants was carried out according to the procedure adopted in the Selection Laboratory of the USSR Antibiotics Research Institute, Moscow. This procedure consists of the following steps:

(1) Three hundred to 500 or more variants, obtained after treatment with mutagenic factors, are tested for their activity by growing them in submerged conditions. Every variant is grown only once, with no parallel run.

(2) The best variants, selected after the first test, are subjected to a sec-

ond test, which is run in duplicate. The most productive variants from this test are left for further tests.

(3) Finally, the selected variants are tested in a series of fermentations with 4 or 6 parallel runs in comparison to the initial strain. As a result of this test,

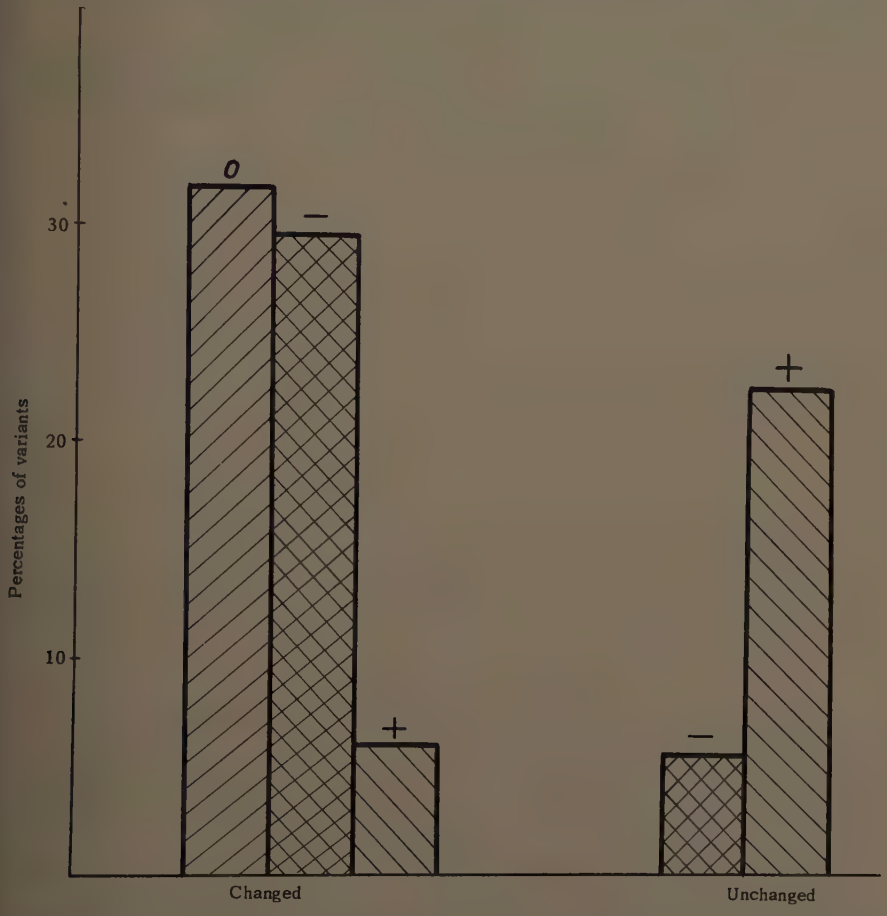

FIGURE 7. The relation between the morphologic features of the variants and their antibiotic activity. Key: O, inactive variants; −, slightly active variants; +, plus variants.

one or two variants, exceeding by their activity the initial strain by at least 10 to 15 per cent and stable in this respect on subsequent transfers, are left.

In case the activity of the newly selected strain is considerably higher than that of the initial one (not less than 20 to 25 per cent), it is tested at first in pilot-plant conditions and then in plant conditions. If the activity of the new strain is not much higher than that of the initial one, it is again subjected to treatment with mutagenic factors, followed by the three-step selection.

To obtain highly active strains, it is more often necessary to repeat the selection process several times. In some cases a considerable increase of the activity of the strains is achieved in a comparatively short period.

Selection of Active Strains Producing Chlortetracycline

The selection of *S. aureofaciens* was begun from strain No. 77. The average activity of this strain was 600 U./ml.* A water spore suspension of strain No. 77 was irradiated with ultraviolet rays in a dose of 3500 erg/sq. mm. Two strains, strains No. 536 and No. 546, were isolated from 776 colonies grown from irradiated spores.[1] The average activity of these strains was 1000 U./ml. It was found that the activity of strain No. 536 was a little higher than that of the other strain when the NH_4Cl in the medium was replaced by NH_4NO_3. Therefore strain No. 536 was chosen for further selection.

Spores of this strain were treated twice with ultraviolet rays with intermediate treatment by visible light. The scheme of the spore treatment was as follows:

ultraviolet rays, 3500 ergs/sq. mm. → visible light,
3 hours → ultraviolet rays, 3500 ergs/sq. mm.

Colony No. 112 was isolated from 1220 colonies. The average activity of this colony was 1200 U./ml. The spores of strain No. 112 were then subjected to combined treatment of ethyleneimine and ultraviolet rays according to the following scheme:

ethyleneimine, diluted → ultraviolet rays
1:5000, 24 hours 4000 erg/sq. mm.

From 1170 colonies grown from these spores strain No. 15 was isolated. The average activity of this strain was 1400 U./ml.

The spores of strain No. 15 were treated with X rays at the dose of 100,000 r and then subjected to combined irradiation with X rays and ultraviolet rays in the following succession:

X rays → ultraviolet rays
36,000 r 6000 erg/sq. mm.

Active strain No. 205 was selected in the experiment in which the spores of strain No. 15 were treated with X rays only. The other strain (No. 134) was selected in the experiment where combined treatment with X rays and ultraviolet rays was used. A total of 2046 colonies was tested in both experiments. The activity of both variants was 1700 U./ml.

The last stage of the selection was isolation of the best variant from 220 colonies grown from the spores of strain No. 134. The spores were treated by X rays and ultraviolet rays. The succession and the doses were the same as in the previous experiment. Strain No. 16 appeared to be the best variant. The average activity of this strain was 2200 U./ml. The activity and morphology of this strain differed markedly from those of the initial strain. Colonies of strain No. 16 were sandy yellow with a greenish tint. The strain synthesized 2500 U./ml. of tetracycline when NaCl in medium was replaced by NaBr in the amount of 0.2 to 0.4 per cent. NH_4CNS in the ammount of 0.1 to 0.05 per cent also was added to the medium. The scheme of breeding of strain LS-B16 is presented in FIGURE 8. As a result of the selection, the activity of the initial strain was increased by 260 per cent.

* The fermentation was carried out in the following medium: corn-steep liquor, 0.5 per cent (dry weight); NH_4NO_3, 0.5 per cent; NaCl, 0.2 per cent; starch, 2.5 per cent; $CaCo_3$, 0.4 per cent; *p*H, 6.4 to 6.6. A rotary shaker at 220 to 240 rpm was used.

Selection of Active Strains Producing Oxytetracycline

The selection scheme of active variants in *S. rimosus* is presented in FIGURE 9. As a starting material, strain 8229 of *S. rimosus* yielding 1500 to 2000 U. of oxytetracycline/ml. of the medium containing corn-steep liquor and starch was used.* When grown on the agarized corn-steep medium this strain forms typical colonies, characterized by light-brown substrate mycelium and white

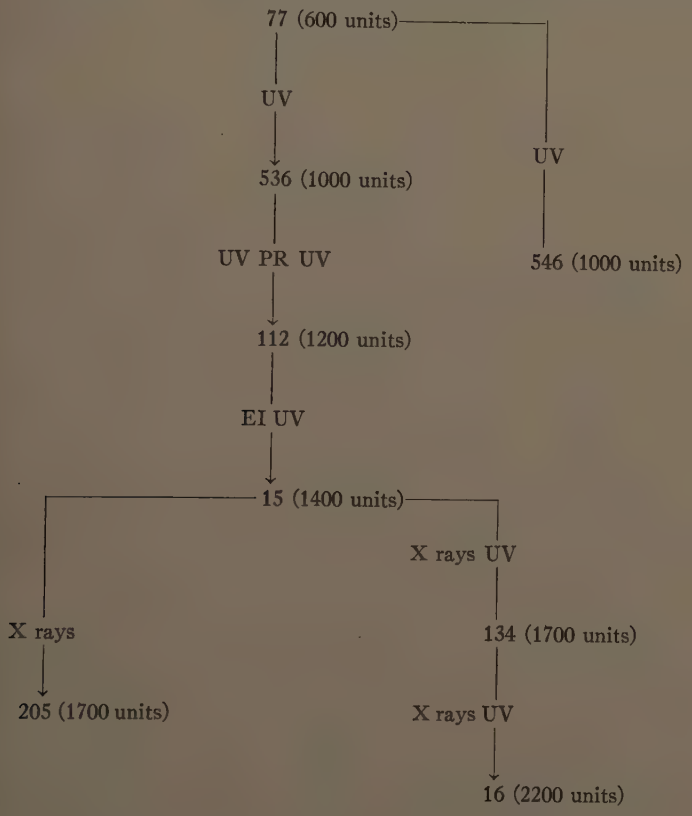

FIGURE 8. The selection scheme of active variants of *S. aureofaciens*. Key: PR, photoreactivity; EI, ethyleneimine; UV, ultraviolet light.

aerial mycelium, chapping with age. Ultraviolet light was used as a mutagenic factor in the selection of active variants.

At the first stage of the selection, strain 1366 with an activity of 2200 to 2600 U./ml. was obtained among 500 variants of strain 8229. Unlike strain 8229, the new one formed folded asporogenic colonies with dark brown substrate mycelium, excreting a buff brown pigment.

At the second stage of the selection, strain LS-T118 with an activity of 2600–3300 U./ml. was obtained as a result of testing 300 ultraviolet-induced vari-

* Composition of medium: corn-steep liquor, 0.25 to 0.5 per cent (dry weight); starch, 3 per cent; $(NH_4)_2SO_4$, 0.4 per cent; NaCl, 0.5 per cent; $CaCO_3$, 0.5 per cent; *p*H, 7.0 to 7.2.

ants. Strain LS-T118 is characterized by poorly sporulating, folded colonies with dark brown substrate mycelium and light gray aerial mycelium, which differ from the colonies of both the initial strain 8229 and strain 1366. In contrast to these strains, the foaming of the fermentation broth inoculated with strain LS-T118 is far less.[6] The comparative activities of strains 8229, 1366, and LS-T118 are presented in TABLE 6.

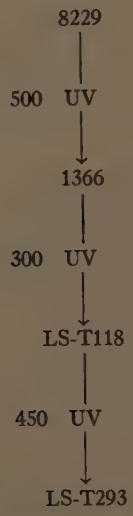

FIGURE 9. The selection scheme of active variants of *S. rimosus*. UV represents ultraviolet light.

TABLE 6
COMPARATIVE ACTIVITY OF STRAINS 8229, 1366, AND LS-T118 OF *S. RIMOSUS*

Strain	Number of runs	Average maximum activity (mg./ml.)	The ratio to the activity of strain 8229 (percentages)	The ratio to the activity of strain 1366 (percentages)
8229	28	1828	100.0	
1366	41	2707	131.7	100.0
LS-T118	40	3049	166.8	121.6

At the third stage of the selection, strain LS-T293 was obtained among 450 variants of strain LS-T118. Almost no difference is observed between these two strains with respect to their morphologic features and antibiotic activity. However, both strains differ markedly in their nutrient requirements. Like other strains of *S. rimosus*, strain LS-T118 produces maximum amounts of the antibiotic in the medium containing 0.25 to 0.5 per cent of corn-steep liquor (depending on the batch). When the amount of corn-steep liquor in the medium is increased to 0.75 to 1.0 per cent, the activity of this strain markedly decreases, whereas strain LS-T293 yields the greatest amounts of the antibiotic in the medium containing 0.5 to 1.0 per cent of corn-steep liquor (FIGURE 10).

This difference between strains LS-T118 and LS-T293 was found to be re-

lated to their different requirements in the soluble inorganic phosphorus present in corn-steep liquor.[13] The requirement of strain LS-T293 for the soluble inorganic phosphorus is three times higher than that of strain LS-T118, 7 to 9 mg. per cent and 2 to 4 mg. per cent, respectively. The conditions optimal for strain LS-T118 appear to be unfavorable for strain LS-T293. In these conditions, strain LS-T293 is characterized by poor growth, slow carbohydrate

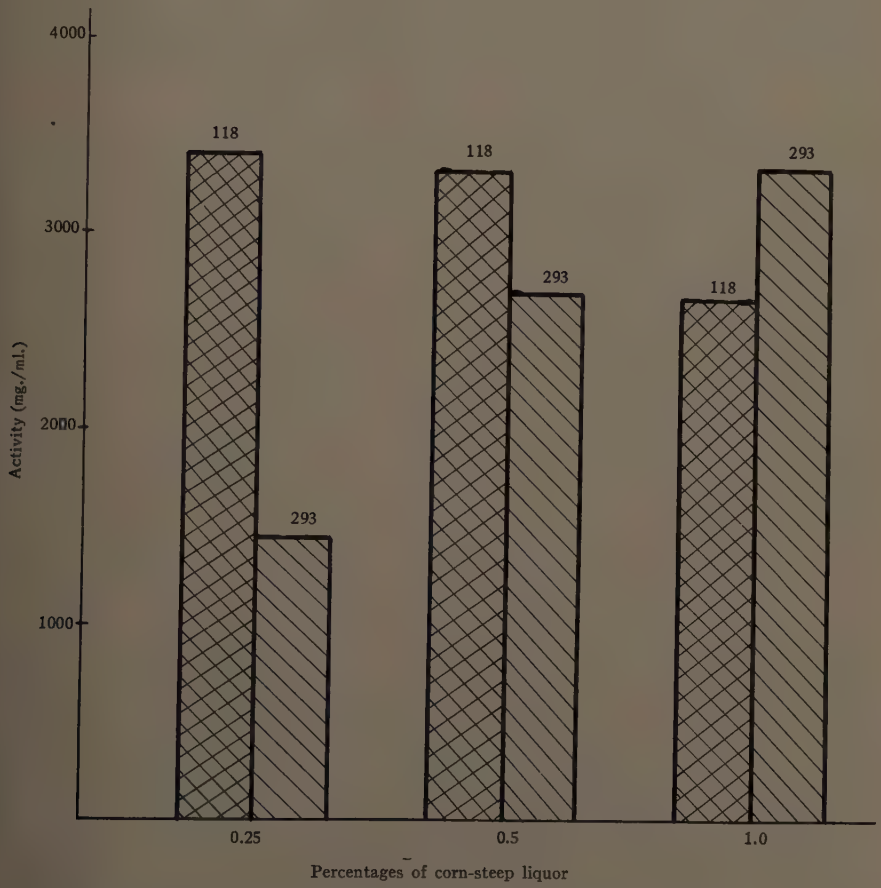

FIGURE 10. The comparative antibiotic activity of strains LS-T118 and LS-T293 as dependent upon the amount of corn-steep liquor in the medium.

utilization, and low antibiotic production levels. Only when the amount of corn-steep liquor in the medium is increased to 0.5 to 1.0 per cent does strain LS-T293 grow well, quickly utilizing carbohydrates and producing the same or a higher amount of oxytetracycline as compared to that of strain LS-T118 in conditions optimal for the latter.

It is necessary to point out that at the third stage of the selection, as a result of which strain LS-T293 was obtained, the corn-steep liquor used contained high amounts of inorganic phosphorus. Thus, the properties of strain LS-T293 were defined by the selection conditions.

The activity of strain LS-T118 in pilot-plant conditions appeared to be more than twice as great as that of strain 8229.

Thus, as a result of the multistage selection among comparatively few ultraviolet-induced variants, a considerable increase of the oxytetracycline yield was achieved.

Discussion

In the majority of published works dealing with the use of mutagenic factors for the selection of antibiotic-producing microorganisms, only the factors used and the results obtained are described. A number of problems very important for successful radioselection is disregarded.

At the same time the antibiotic-producing microorganisms are shown to be the biological forms in which the possibility of a practical use of mutagenic factors (such as UV rays and X rays, nitrogen mustard, and ethyleneimine) is demonstrated convincingly. Therefore, it seems to us that to use these factors in the selection of antibiotic producers with more success, a number of investigations is necessary.

The problem of the most effective methods for application of mutagenic factors in the selection of microorganisms is not yet solved. In our experiments we used UV rays and X rays, ethyleneimine, and their combinations. On the basis of the data obtained, it is very difficult to give preference to this or that factor or to their combination. It may be assumed only that the combined action of mutagenic factors may increase considerably the effectiveness of selection.

The choice of the dose of one or another factor is a problem of great importance. As is evident from our data, a dose optimal for the formation of plus variants does not coincide with the dose optimal for the formation of minus variants. Further studies in this direction are also necessary.

The third essential problem concerns the correlation between the change of morphologic features, on the one hand, and antibiotic-producing ability, on the other. As shown by us in *S. rimosus*, the majority of morphologic mutants are characterized by a lower activity, whereas the majority of variants with a higher activity do not differ markedly in their morphologic features from typical colonies. However, a conclusion about the existence of correlation between morphologic features and antibiotic activity based on these facts would be erroneous, especially as it concerns the possibility of selection on the ground of morphologic features. First, the division on unchanged and changed colonies is relative, for only variants with a marked difference from typical colonies were regarded by us as belonging to changed variants. Second, among morphologically changed colonies we also observed more active variants. Third, highly active strains, as a rule, differ markedly in their morphologic features from strains with low activity. Therefore, in most cases the morphologic peculiarities of colonies can not serve as a criterion of their activity. Hence, while selecting more productive strains, it is necessary to determine the activity of all, or almost all colonies, neglecting only obviously degenerative forms.

The fourth problem is the problem of utilization of great mutations. A change of only one feature with the retention of others in microorganisms, as well as in higher plants, may be very useful for increasing their productivity.

A case of obtaining a pigmentless penicillin-producing strain is described in the literature. We obtained a similar mutation (phosphorus) in *S. rimosus*. Strain LS-T293, which emerged as a result of this mutation, appeared to be adapted to high concentrations of corn-steep liquor in the medium.

The achievements attained in the radioselection of antibiotic-producing microorganisms are obvious. The effectiveness of mutagenic factors in selection is beyond doubt. However theoretical studies on the problems of genetics and selection of microorganisms are still lagging behind their practical application.

Recombinations in *S. rimosus*

Genetic recombinations are described at present in several species of actinomycetes, particularly in *S. coelicolor*, *S. fradiae*, and *S. griseoflavus*.[14-18] This permits one to consider the process of recombination formation among actinomycetes to be widespread.

We have obtained recombinations in one more species of actinomycetes, namely, in *S. rimosus*.[19, 20] The latter is the producer of oxytetracycline; partially because of this, it was used to study the recombination processes in actinomycetes. In addition to the morphologic and biochemical peculiarities of recombinants, their stability, and their frequency of emergence, we were interested also in studying their antibiotic activity as compared with the activity of their parent forms. As is known, first attempts to use hybridization for selection of imperfect fungi used in industry had given positive results.[21, 22] In this connection the possibility of using recombinations in the selection of antibiotic-producing actinomycetes had arisen.

Materials and Methods

For our experiments we used 4 strains of *S. rimosus*, namely, strains 101, 8229, LS-T293, and BS-21. All of these strains are active producers of oxytetracycline. Among them, strain LS-T293 obtained by us from strain 8229 as a result of ultraviolet selection was the most active.

In all four strains biochemical mutations were induced by irradiating their spores with ultraviolet rays. As the complete medium, a medium with corn-steep liquor was used; as the minimal medium, a modified Czapek medium. Isolation of biochemical mutants was carried out in most cases according to the modified method of Beadle and Tatum[23] and, in some cases, according to the replica-plating method of Lederberg, as modified by Szybalski.[14, 24] Obtaining recombinants was carried out according to the ordinary scheme.[14, 17] The agarized complete medium in Petri dishes was inoculated with a mixture of spores (or mycelial fragments, if the spores were not available) of 2 biochemical mutants cultivated separately. After 7 to 8 days' incubation at 27 to 28° C. the spores were washed off the mixed growth surface with sterile distilled water. The suspension obtained was filtered and then plated upon the complete and the minimal media. At the same time a mixture of suspensions of biochemical mutants grown separately was plated upon the minimal medium for control.

The growth of prototroph colonies in the minimal medium was observed only in the case of preliminary mutual cultivation of biochemical mutants in the complete medium. The frequency of prototrophs was determined from the

proportion between their number and the number of colonies grown in the complete medium (the dilution was taken into account). It must be noted that this method allows one to determine not so much the frequency of recombinant emergence as the proportion between the total amount of spores (and small fragments of hyphae) and the amount of recombinant spores that are present in the suspension.

When double auxotrophic mutants were used for obtaining recombinations, the spore suspension from the mixed growth was plated, not only on the minimal medium, but also on the minimal medium enriched with growth factors necessary for these biochemical mutants. In this case we chose only those combinations of growth factors that could stimulate only the growth of auxotrophic recombinants and could not stimulate the growth of the initial biochemical mutants. To study the antibiotic activity of recombinants, biochemical mutants and initial strains of *S. rimosus*-submerged fermentation was carried out in shake flasks. For this purpose a medium with corn-steep liquor was used. The antibiotic activity was determined by the agar diffusion method with test microbe L2 (*B. subtilis*) on the fourth or fifth day of fermentation.

A more detailed description of methods for obtaining biochemical mutants and recombinants, as well as a chemical comparison of media and cultivation conditions necessary for working with *S. rimosus*, is given in our previous papers.[20, 25, 26]

Results

Obtaining Biochemical Mutants

A total of 59 identified biochemical mutants was obtained from the 4 *S. rimosus* strains. All these mutants except 2 were selected after single irradiation with ultraviolet light; therefore, they may be considered as single biochemical mutants, that is, as mutants carrying 1 factor of biochemical deficiency. The 2 biochemical mutants were obtained as a result of ultraviolet irradiation of single biochemical mutants, and therefore they may be considered as double biochemical mutants, that is, as mutants carrying 2 factors of biochemical deficiency. Of 57 single biochemical mutants, 4 biochemical mutants were obtained from strain 101, 16 from strain 8229, 16 from strain BS-21 and 21 from strain 293. All of the single biochemical mutants are listed in TABLE 7. From this table it is evident that mutants requiring histidine, isoleucine, and valine, as well as vitamin P-P and methionine (or cystine), were more frequent. Biochemical mutants with alternative requirements also were frequent. For example, there was a mutant obtained from strain BS-21 that required histidine, proline, or glutamic acid, as well as a mutant obtained from strain 293 that required one of the following amino acids: arginine, tryptophan, histidine, serine, or norleucine.

The frequency of biochemical mutants was different in different strains and experiments, ranging from 0.25 to 1.1 per cent. The overwhelming majority of the biochemical mutants differed from the initial strain of *S. rimosus* both in the biochemical and the morphologic properties, namely, in the colony shape and size, the sporulation level, the color of the substrate mycelium, and the amount of the pigment excreted. Very often the biochemical mutants formed

asporogenic colonies and were characterized by a lower growth rate as compared to the initial strain.

Obtaining Recombinants and Their Morphologic Properties

To obtain recombinants, two biochemical mutants were grown together in various combinations. For composing the combinations, those biochemical mutants in which very little or no synthropic growth was observed on mixed cultivation in the minimal medium were used.

TABLE 7

NUTRIENT REQUIREMENTS OF THE IDENTIFIED BIOCHEMICAL MUTANTS OF *S. RIMOSUS*

Growth factors required	Number of mutants obtained from the following strains				Total mutants	
	101	8229	LS-T293	BS-21	Number	Percentages
Histidine	3	2	11	2	18	31.6
Valine + isoleucine	—	2	1	4	7	12.3
Methionine + threonine	—	4	—	—	4	7.0
Arginine	1	1	—	1	3	5.2
Arginine/ornithine	—	—	1	3	4	7.0
Methionine	—	1	—	—	1	1.8
Methionine/cystine	—	—	3	—	3	5.2
Vitamin P-P	—	2	1	3	6	10.5
Threonine	—	2	—	—	2	3.5
Ornithine/proline	—	—	—	—	1	1.8
Vitamin B_2	—	—	1	—	1	1.8
Leucine	—	—	—	1	1	1.8
Lysine	—	1	—	—	1	1.8
Asparagine + leucine	—	1	—	—	1	1.8
Glycine/lysine/serine	—	—	1	—	1	1.8
Arginine/tryptophan/histidine/serine/norleucin	—	—	1	—	1	1.8
Histidine/arginine/glycine/phenylalanine	—	—	1	—	1	1.8
Histidine/proline/glutamic acid	—	—	—	1	1	1.8
Total number of biochemical mutants	4	16	21	16	57	100

The recombinations did not occur in every combination of the biochemical mutants. Of 76 combinations tested, the recombinants were obtained only in 17 combinations. It is interesting that recombinations were formed between the biochemical mutants either of the same strain or of the different ones, the frequency of their formation from different strains being not lower than that from the same strain.

Of 39 combinations of the biochemical mutants obtained from different strains, the recombinations were observed in 9 combinations (23 per cent) whereas, of 37 combinations of biochemical mutants obtained from the same strain, the recombinations were observed in 8 combinations (21.6 per cent).

The frequency of prototrophic recombinations varied greatly, depending on the combination of the biochemical mutants used (TABLE 8). Thus, it was 2×10^{-4} in the combination 101-870-2 hist, val + isl × 8229-1361-1 met + thr;

in the combination 8229-310 val + isl × 8229-1361 thr, the recombination frequency was about 500 times lower, or 4×10^{-7}. Consequently, the absence of recombinants in many combinations of the biochemical mutants may be accounted for by very low frequency of their formation. In fact, when the density of the suspension is 1×10^{7} per 0.1 ml. and the frequency of recombinations is lower than 1×10^{-7}, it is very difficult to detect the recombinants.

The chief pecularity of the prototrophic recombinations in *S. rimosus* emerging from the same combination of biochemical mutants is their marked difference with respect to their morphology. As a rule, the prototrophs obtained from every combination of biochemical mutants belong to 2 or 3 types. One or 2 of them usually are characterized by well-sporulating colonies, while the others form asporogenic colonies. It is of interest that the prototrophs in 1 combination of biochemical mutants obtained from 2 strains of *S. rimosus* differ, as a rule, from the prototrophs in another combination of biochemical

TABLE 8

FREQUENCY OF PROTOTROPHS IN DIFFERENT COMBINATIONS OF BIOCHEMICAL MUTANTS OF *S. RIMOSUS*

Biochemical mutant combination	Number of spores planted on minimal medium	Number of colonies grown	Frequency of prototrophs
101-870 hist × 8229-1361 thr*	6.4×10^{8}	391	6×10^{-5}
101-870 hist × 8229-310 val + isl	4×10^{7}	122	3×10^{-6}
8229-310 val + isl × 8229-1361 thr	7.2×10^{7}	31	4×10^{-7}
101-870-2 hist, val + isl × 8229-1361-1 met + thr	2.5×10^{5}	45	1.8×10^{-4}
101-870-2 hist, val + isl × 8229-1361-1 a met + thr, a	2.6×10^{6}	6	2.3×10^{-6}
293-1 val + isl × BS21-24 arg	4.4×10^{8}	439	7×10^{-6}
293-1 val + isl × BS21-49 hist	3×10^{6}	24	8×10^{-6}

* The first figure refers to the number of the initial strain; the following figures refer to the numbers of mutants. Key: hist, histidine; val, valine; isl, isoleucine; met, methionine; thr, threonine; arg, arginine; leu, leucine; a, unidentified growth factor present in corn-steep liquor.

mutants belonging to the same strains. Moreover, all of these prototrophs differ from both initial strains. The same phenomenon was observed when 2 biochemical mutants obtained from 1 strain of *S. rimosus* were used. For example, in the cross between biochemical mutants of strains 101 and 8229, that is, in the cross 2a-870 hist × 8229-310 val + isl, 2 types of prototrophs were usually observed: prototrophs with yellowish sporulating colonies and prototrophs with smooth asporogenic colonies, characterized by black substrate mycelium. In the cross between the same mutant of strain 101 and another mutant of strain 8229, cross 2a-870 his × 8229-1361 thr, the prototrophs obtained were also of 2 types: prototrophs with white sporulating colonies and prototrophs with folded asporogenic colonies characterized by black substrate mycelium. Thus, in the 2 crosses where the same biochemical mutant was used as 1 parent and 2 different biochemical mutants of the same strain as the other, various types of prototrophic recombinants were observed.

In addition to crosses between single biochemical mutants, 2 crosses between double biochemical mutants were studied: crosses 2a-870-2 his; val + isl ×

8229-1361-1 thr + met and 2a-870-2 his; val + isl × 8229-1361-1 a thr + met; a. In these crosses one and the same biochemical mutant was used as 1 parent and 2 mutants of strain 8229 differing by 1 factor of biochemical deficiency, factor a, were used as the other parent. In both crosses prototrophs of 3 or 4 types were obtained, the 2 which were observed more frequently being the same in both crosses. One of these types was characterized by white sporulating colonies and the other by dark-brown smooth asporogenic colonies.

Besides prototrophic recombinants, two types of auxotrophic ones were also observed in these crosses: auxotrophs requiring histidine and auxotrophs requiring valine and isoleucine (TABLE 9).

In common with the prototrophs, morphologically different forms were observed among the auxotrophs.

During the examination of the recombinants from the combination 101-870-2 hist, val + isl × 822-1361-1 met + thr, a phenomenon that seems also to be

TABLE 9

OCCURRENCE OF PROTOTROPHIC AND AUXOTROPHIC RECOMBINANTS IN CROSSES BETWEEN DOUBLE BIOCHEMICAL MUTANTS OF *S. RIMOSUS*

Combination	Amino acid added to minimal medium	Recombination frequency	Number of colonies studied	Recombinants obtained	
				Type	Number
101-870-2 hist; val + isl × 8229-1361-1 met + thr	Valine and isoleucine	4.1×10^{-4}	36	val + isl +	16 20
	Histidine	2.4×10^{-4}	29	hist +	2 27
101-870-2 hist; val + isl	Valine, isoleucine, methionine, threonine	2.5×10^{-5}	36	val + isl +	35 1
× 8229-1361-1 a met + thr; a	Histidine, methionine, threonine	4.7×10^{-6}	6	hist +	5 1

characteristic of other crosses was observed. On plating the spores of prototrophic colonies grown at first on the minimal medium and then transferred to the complete medium, several types of recombinants often were observed. In one experiment 3 such colonies were obtained of 15 colonies studied. Two of these colonies gave rise to 3 recombinant types, 2 of which proved to be prototrophs of Types I and II, and the third one proved to be an auxotroph requiring valine and isoleucine. One colony gave rise to 2 recombinant types, that is, to prototrophs of Type III and auxotrophs requiring valine and isoleucine.

We are not absolutely certain that these colonies arose from single spores. To prove this assumption it is necessary to isolate the spores by means of a micromanipulator. If the above-mentioned colonies did not arise from single spores but from spore chains or short mycelial fragments, it should be necessary to assume that the nearby nucleoids gave rise to various types of recombinants.

In this connection it is interesting to mention that Braendle and Szybalski described cases in which colonies arose from a single conidium or a filtering

mycelial fragment and consisted of a mixture of conidia belonging to the two parent types and two recombinant types.[14] According to Bradley (personal communication) some spores of *S. coelicolor* contain two or more genomes.

Study on the Antibiotic Activity of Recombinants, Their Parents, and Initial Strains

Antibiotic activity of initial strains of S. rimosus. As already mentioned, all strains of *S. rimosus* used in the present work are active producers of oxytetracycline. With respect to the increment of their activity (if they are cultivated in a corn-steep liquor medium) they were arranged as follows:*

Strain No. 101 with the activity of 1200–1500 mg./ml.
Strain No. 8229 with the activity of 1400–2000 mg./ml.
Strain BS-21 with the activity of 1800–2200 mg./ml.
Strain LS-T293 with the activity of 2600–3200 mg./ml.

In contradiction to other strains, strain LS-T293 grew well and produced much antibiotic only at high concentrations of soluble inorganic phosphorus in the medium.

Therefore, the optimal concentration of corn-steep liquor possessing inorganic phosphorus for this strain is 0.5 to 1.0 per cent (dry weight), whereas the 3 strains gave best results when the fermentation was conducted in a medium with 0.25 to 0.5 per cent of corn-steep liquor.

Antibiotic activity of biochemical mutants. All biochemical mutants independent of strains from which they are derived can be divided, according to their activity, into 3 groups (TABLE 10). The first group consists of biochemical mutants with very low antibiotic activity (less than 25 per cent of the activity in the control) or without activity at all. This group included mutants requiring histidine, arginine (or ornithine), leucine, valine, and isoleucine, as well as some other mutants. The activity of these biochemical mutants increases slightly or remains the same when growth factors required by the mutants are added to the fermentation medium. The second group consists of mutants requiring methionine (or cystine), mutants requiring vitamin P-P (or niacin), and a mutant requiring vitamin B_2. All these mutants, as well as the mutants of the first group, produce little antibiotic in the ordinary fermentation medium with corn-steep liquor. However, when methionine, 100 μg./ml. (for methionine-requiring mutants), niacin, 10 μg./ml. (for niacin-requiring mutants), or vitamin B_2, 20 μg./ml. (for vitamin B_2-requiring mutants), is added to the medium, the activity of these mutants markedly increases and reaches that of the initial strain. The third group consists of 2 mutants of strain No. 293 and 1 mutant of strain BS-21 with the activity of more than 50 per cent of the initial strain activity.

As the data in TABLE 10 show, mutants possessing synonymous factors of biochemical deficiency are characterized by almost identical antibiotic activity. For example, all 6 mutants requiring valine and isoleucine and 16 of the histidine-requiring mutants produce almost no antibiotic. On the other hand, all

* The composition of the medium used for all laboratory fermentations (in 300-ml. flasks, shaken at 220 to 240 rpm) was as follows: corn-steep liquor, 0.25 to 0.5 per cent (dry weight); starch, 3.0 per cent; $(NH_4)_2SO_4$, 0.4 per cent; NaCl, 0.5 per cent; $CaCo_3$, 0.5 per cent; *p*H, 7.0 to 7.2.

4 methionine and 5 niacin mutants produce in the ordinary fermentation medium not more than one third of the amount of antibiotic produced by the initial strain in the same fermentation medium. With the addition of specific growth factors, their activity reaches that of the initial strain and, in some cases, exceeds it. The activity of Group II mutants was studied more thoroughly.[26] One mutant requiring methionine (mutant 8229-1125a) and one mutant requiring niacin (mutant 8229-925) were taken. The activity and dry weight of mycelium of these mutants in corn-steep liquor medium with and

TABLE 10
RELATION BETWEEN THE NUTRIENT REQUIREMENTS OF THE MUTANTS AND THEIR ANTIBIOTIC ACTIVITY

Growth factors required	Number of biochemical mutants	Antibiotic activity ranges of biochemical mutants (as percentages of the activity of the initial strain)	
		Without growth factors added	With growth factors added
Histidine	16	0–17*	—
Valine + isoleucine	6	1–7	—
Methionine + threonine	4	2–3†	—
Arginine	3	0–1	—
Arginine/ornithine	4	0–5	—
Ornithine/proline	1	22	—
Threonine	2	2–4	—
Leucine	1	4	—
Lysine	1	0	—
Asparagine + leucine	1	7	—
Glycine/lysine/serine	1	0	—
Methionine/cystine	4	4–28	65–92
Vitamin P-P	5	8–28	93–144
Vitamin B_2	1	4	88
Arginine/tryptophan/histidine/serine + norleucine	1	81	—
Histidine/arginine/glycine/phenylalanine	1	73	—
Histidine/proline/glutamic acid	1	52	—

* Histidine-requiring mutant with activity of about 60 per cent of the activity of the initial strain is not included. The probability of back mutations in this case is not excluded.

† Methionine- and threonine-requiring mutant with activity of about 40 per cent of the activity of the initial strain is not included. In this case also the probability of back mutations is not excluded.

without addition of corresponding growth factors were studied in comparison with the activity and the dry weight of the initial strain. The data given in TABLE 11, which presents the results of a typical experiment, show that the sharp increase in the antibiotic activity of both mutants is not connected with any considerable change in their mycelium dry weight when methionine and niacin are added.

At the same time the antibiotic activity and dry weight of the initial strain were not changed markedly when methionine and niacin are added to the corn-steep liquor medium.[26]

The experiments with the fermentation of methionine and niacin mutants

in a synthetic medium with different concentrations of corresponding growth factors confirmed the fact that the maximum production of antibiotic by these mutants requires growth factors in amounts greater than those necessary for their normal growth.[26]

Consequently, the low activity of methionine and niacin mutants in the corn-steep liquor medium is explained by the fact that the amounts of methionine and niacin in such a medium are sufficient to provide good growth of both mutants. However, this amount is not sufficient for the realization of

TABLE 11

ACTIVITY AND GROWTH OF *S. RIMOSUS* STRAIN 8229 AND ITS BIOCHEMICAL MUTANTS ON CORN-STEEP MEDIA WITH AND WITHOUT CORRESPONDING GROWTH FACTORS ADDED

Strain	Maximum antibiotic activity (mg./ml.)		Mycelium dry weight (mg. per cent)	
	No growth factors added	Growth factors added	No growth factors added	Growth factors added
8229	1982	—	884	—
8229-1125 met	560	1837	915	906
8229-995 ni	90	2031	718	756

TABLE 12

FREQUENCY OF RECOMBINATIONS BETWEEN BIOCHEMICAL MUTANTS 101-870 HIST AND 8229-310 VAL + ISL AS DEPENDENT UPON THE TIME OF MIXED GROWTH (RATIO 1 "870":180 "310")

Time of mixed growth (days)	Plating of spores			Plating of mycelium		
	Total number of spores plated	Number of colonies grown	Prototroph frequency	Total number of fragments plated	Number of colonies grown	Prototroph frequency
3	3.5×10^5	0	3×10^{-6}	1×10^5	0	9×10^{-6}
4	2.0×10^5	0	5×10^{-6}	6.5×10^4	0	1.5×10^{-5}
5	3.4×10^5	2	6×10^{-6}	4.9×10^5	0	2×10^{-6}
6	7.0×10^5	81	1.1×10^{-4}	1.8×10^5	0	5×10^{-6}
8	10.4×10^5	667	6.4×10^{-4}	1.4×10^6	101	7.2×10^{-5}
10	10.3×10^5	970	9.3×10^{-4}	5.6×10^4	46	8.2×10^{-4}

the ordinary process of oxytetracycline biosynthesis. On the basis of the data obtained it is impossible to conclude that these substances actually take part in the process of oxytetracycline biosynthesis.

Antibiotic Activity of Recombinants

The antibiotic activity of prototroph recombinants from seven different crosses between biochemical mutants was studied. In six crosses the biochemical mutants were obtained from various strains of *S. rimosus*.

In all cases the activity of prototrophs from one and the same cross varied markedly, as did their morphologic features. It is interesting to note that colonies similar in their morphology were also similar in their antibiotic activ-

ity. Therefore the activity of only one colony from each morphologic type of prototroph from the given combination usually was studied.

Tables 13 and 14 present the data on the antibiotic activity of prototroph recombinants obtained from crosses between single and double biochemical

Table 13

Comparative Antibacterial Activity of the Initial Strains of *S. rimosus*, Their Biochemical Mutants and Prototrophs

Strain	Antibacterial activity (mg./ml.)
Initial strains	
8229	1429
101	1234
Biochemical mutants	
8229-1361 thr	262
8229-310 val + isl	97
101-870 hist	28
Prototrophs from combination 101-870 hist × 8229-310 val + isl	
Type I	705
Type II	1622
Type III	1555
Prototrophs from combination 101-870 hist × 8229-1361 thr	
Type I	663
Type II	281
Type III	1269

Table 14

Comparative Antibacterial Activity of the Initial Strains of *S. rimosus*, Their Double Biochemical Mutants and Prototrophs

Strain	Antibiotic activity (mg./ml.)
Initial strains	
101	1575
8229	1670
Double biochemical mutants	
101-870-2 hist; val + isl	76
8229-1361-1 met + thr	1.0
8229-1361-1 a met + thr; a	0
Prototrophs from combination 101-870-2 hist; val + isl × 8229-1361-1 met + thr	
Type I	747
Type II	867
Prototrophs from combination 101-870-2 hist; val + isl × 8229-1361-1 a met + thr; a	
Type I	640
Type II	1017

mutants of strains 101 and 8229. As is evident from these tables, the activity of almost all these prototrophs is much higher than that of the biochemical mutants. Moreover, some of them produce the same or even a greater amount of antibiotic than the initial strains. Such strains were obtained only from crosses between single biochemical mutants.

TABLE 15 presents the results of studying the antibiotic activity of recombinants obtained from 2 crosses between biochemical mutants of strains LS-T293 and BS-21. These strains, as mentioned previously, are characterized by different requirements in inorganic phosphorus. Therefore, to test the activity, 2 variants of the fermentation medium containing 0.25 and 0.75 per cent of corn-steep liquor were used in this case.

As is evident from TABLE 15 the activity of strain BS-21 in both media was almost the same (it must be noted that the activity of this strain in a medium containing 0.25 per cent of corn-steep liquor is usually a bit higher than in a medium with 0.75 per cent of corn-steep liquor), whereas the activity of strain

TABLE 15

COMPARATIVE ACTIVITY OF STRAINS LS-T293 AND BS-21 OF *S. RIMOSUS*, THEIR BIOCHEMICAL MUTANTS AND RECOMBINANTS IN MEDIA CONTAINING DIFFERENT AMOUNTS OF CORN-STEEP LIQUOR

Strain	Antibacterial activity (mg./ml.)		Ratio of the activity in 0.25 per cent corn-steep liquor media to the activity in 0.75 per cent corn-steep liquor media
	Media with 0.25 per cent of corn-steep liquor	Media with 0.75 per cent of corn-steep liquor	
Initial strains			
LS-T293	1775	2650	149
BS-21	1800	1900	106
Biochemical mutants			
293-1 val + isl	7	4	
BS-21-49 hist	27	115	
BS-21-24 arg	0	0	
Prototrophs from combination 293-1 val + isl × BS-21-49 hist			
Type I	1125	2025	180
Type II	2925	1850	63
Type III	1700	1150	68
Prototrophs from combination 293-1 val + isl × BS-21-24 arg			
Type I	1500	23	1.5
Type II	630	61	9.7
Type III	920	1800	195

LS-T293 increased 1.5 times with the increase of corn-steep liquor concentration. As to the prototrophs, the activity of some of them increased with the increase of corn-steep liquor concentration, whereas the activity of others fell sharply. Such prototrophs were observed in both combinations studied.

Prototrophs of Type II obtained from the combination 293-1 val + isl × BS-21-49 hist are of particular interest. The activity of this prototroph is higher, to a certain extent, than that of strain LS-T293 but, in contradistinction to the latter, this prototroph responds negatively to the increase of corn-steep liquor concentration in the medium. In this respect it resembles the majority of *S. rimosus* strains.[13] It should be noted also that prototrophs of Types I and II from combination 293-1 val + isl × BS-21-24 arg are almost inactive in the medium with 0.75 per cent of corn-steep liquor, although they are active in the medium with 0.25 per cent of corn-steep liquor.

One might assume that such recombinants, so different in their properties, appeared because strains LS-T293 and BS-21, differing both in activity and in requirements in inorganic phosphorus, were taken as initial strains for crossing. However, the data given in TABLE 16 show that recombinants responding differently to the increase in corn-steep liquor concentration may appear as a result of the crossing of biochemical mutants belonging to one and the same strain of *S. rimosus*.

Any conclusions concerning the prospect of the use of the recombination phenomenon for obtaining highly productive strains of *S. rimosus* apparently would be premature at present. Obvious, however, is the fact of the existence of great variation among recombinants according to their antibiotic-producing ability and, even more so, according to their requirements for the composition of the fermentation medium. These facts offer a possibility for the use of recombinants as a material for selection.

TABLE 16

COMPARATIVE ACTIVITY OF STRAIN 8229 AND THE TWO TYPES OF RECOMBINANTS BETWEEN BIOCHEMICAL MUTANTS OF THIS STRAIN IN MEDIA CONTAINING DIFFERENT AMOUNTS OF CORN-STEEP LIQUOR

Strain	Oxytetracycline yield (mg./ml.)		Ratio of activity in 0.5 per cent corn-steep liquor medium to activity in 0.25 per cent corn-steep liquor medium (percentages)
	Percentages of corn-steep liquor in the medium		
	0.25	0.75	
8229	1470	625	43
Recombinant 8229-310 val + isl × 8229-1361 thr of Type II	1600	1145	72
Recombinant 8229-310 val + isl × 8229-1361 thr of Type I	740	1555	210

It is necessary to point out that, in all combinations studied, only inactive biochemical mutants were used as parent forms. In the future we shall study the antibiotic-producing properties of recombinants obtained after crossing active biochemical mutants, such as methionine- and niacin-requiring mutants.

Stability of Recombinants

The stability of the recombinants was studied for 4 or 5 generations. Aerial spores (if there was aerial mycelium) were used for the plating.

On the whole, the stability of 12 different types of prototrophic recombinants from 4 combinations of biochemical mutants belonging to strains 101 and 8229 was studied. Most recombinants (9 types of 12) proved to be significantly stable. They never produced auxotrophic segregants, and few morphologically changed colonies (up to 1 per cent) may account for the natural variation of the recombinants.

However, several prototrophic recombinants appeared to be unstable. Some of them regularly segregated out auxotrophic colonies resembling one of the parent forms, while the other segregated out prototrophic colonies belonging to a different morphologic type.

A regular segregation of auxotrophs was observed in several prototrophs of Types I and II from the combination 8229-310 val + isl × 8229-1361 thr. In common with their parent 8229-1361 thr, these segregants required threonine but differed from it, as well as from one another, in morphologic properties. The frequency of the threonine segregants in both prototroph types varied from 3 to 6 per cent. Strains similar to the other parent in respect to their biochemical properties, that is, similar to the biochemical mutant 8229-310 val + isl, were not detected.

Of 7 prototrophs of Type I tested, only 2 appeared to be unstable, while of 51 prototrophs of Type II, 42 produced segregants. The segregating and non-segregating recombinants of the same type did not differ morphologically, nor was the difference between their activities significant.

Several recombinants of Type IV from the combination 101-870-2 hist, val + isl × 8229-1361-1 met + thr always segregated out prototrophic colonies that did not differ morphologically from recombinants of Type II obtained from the same combination. This segregation was immediately observed since recombinants of Type IV are characterized by pale (cream-colored), much-folded, and asporogenic colonies, while recombinants of Type II also form asporogenic but dark-brown smooth colonies.

Other recombinants of Type IV from the same combination segregated out dark colonies, morphologically different from recombinants of Type II, but also belonging to prototrophs.

Further studies demonstrated that many of the segregants described also were unstable and produced segregants on subsequent transfers. Moreover, both the prototrophic and auxotrophic segregants dissociated only according to their morphologic features. A very complicated dissociation was observed in segregants of Type IV prototrophs from the combination 101-870-2 hist, val + isl × 8229-1361-1 met + thr. These segregants produced colonies of several types. Many of these colonies continued to produce segregants on subsequent transfers.

Among the recombinants studied, we never observed any that segregated out both initial forms, while such recombinants were described by Bradley[27] in *S. coelicolor*.

Recombination Frequency as Dependent upon the Conditions and Time of Mixed Growth of the Parent Forms

In connection with low frequency of recombinations in *S. rimosus*, amounting in some cases to 10^{-7}, the problem of obtaining them appears to be very difficult. Therefore, the searches for the ways of increasing the recombination frequency are of great importance.

Frequency of recombinations as dependent upon the ratio of two biochemical mutants grown together. When beginning the study on the effect of the ratio of biochemical mutant spores in the mixed culture on the recombination frequency, we proceeded from the following premises.

(1) The ratio of two biochemical mutants in the mixture influences the frequency of heterokaryon formation in actinomycetes.[28]

(2) Due to the different growth rates for different biochemical mutants,

their ratio in the mixture must play an important role in creating conditions favorable for the formation of recombinants.

The mixed suspensions were prepared from submerged spores or short mycelium fragments of 2 biochemical mutants. To obtain different proportions of the spores, the starting suspensions were used at various dilutions (1:10, 1:100, 1:1000). The diluted spore suspensions of one mutant were mixed with undiluted spore suspensions of the other mutant, and vice versa. The actual proportions of the spores of the 2 components in the mixture were determined by plating the correspondingly diluted suspensions in Petri dishes with the complete medium and by counting the number of the colonies grown. The recombinant frequency was determined in the usual way on the seventh or eighth day after mixed growth of the two biochemical mutants.

The data on recombination frequency in crosses between biochemical mutants 2a-870 hist and 8229-310 val + isl are presented in TABLE 17 and FIGURE

TABLE 17

FREQUENCY OF RECOMBINATIONS BETWEEN BIOCHEMICAL MUTANTS 101-870 HIST AND 8229-310 VAL + ISL AT THEIR VARIOUS RATIOS IN THE MIXED CULTURE

Experiment No. 1		Experiment No. 2		Experiment No. 3	
Ratio 870:310	Prototroph frequency	Ratio 870:310	Prototroph frequency	Ratio 870:310	Prototroph frequency
1:2	9×10^{-7}	1:1.5	1.9×10^{-6}	1.3:1	4.9×10^{-6}
1:20	2.6×10^{-5}	1:14	1.4×10^{-5}	1:7	8.2×10^{-6}
1:200	9×10^{-4}	1:140	1.8×10^{-3}	1:75	9×10^{-4}
—	—	—	—	1:750	6×10^{-4}
5:1	6×10^{-6}	7:1	3.5×10^{-5}	14:1	5.4×10^{-5}
50:1	8×10^{-5}	70:1	3×10^{-4}	140:1	2.5×10^{-4}
—	—	—	—	1400:1	5.8×10^{-3}

11. Examination of the data presented shows that the recombination frequency increases several hundred times when the proportions of the 2 biochemical mutants in the mixture are markedly different, irrespective of the predominance of this or that component.

Similar results were obtained with other combinations of the biochemical mutants, such as 2a-870-2 hist, val + isl × 8229-1361-1 × met + thr, a.

We used the described phenomenon for obtaining recombinants in crosses characterized by very low frequency of their formation. However, it should be noted that, in crosses with comparatively high recombination frequency amounting to 10^{-3} to 10^{-4}, the use of suspensions with different proportions of spores of the 2 parent types had no effect and the frequency of recombinations remained unchanged.

The marked increases of the recombination frequency with the use of suspensions containing different amounts of spores of the two parent forms may be explained by the fact that, on being plated, each spore or a mycelium fragment of the mutant used in the smaller amount appears to be surrounded by the spores of the mutant used in the greater amount, thus increasing the prob-

ability of heterokaryotic mycelium formation and, consequently, the frequency of recombinations.

This assumption is confirmed by an increase in the recombination frequency with a decrease in the amount of spores of either one parent or the other. It is not contradictory to absence of the effect when different proportions of the parent spores are used in crosses characterized by comparatively high recombination frequency.

In connection with the formation of prototrophic recombinants belonging to several morphologic types in the mixed culture of two biochemical mutants, it

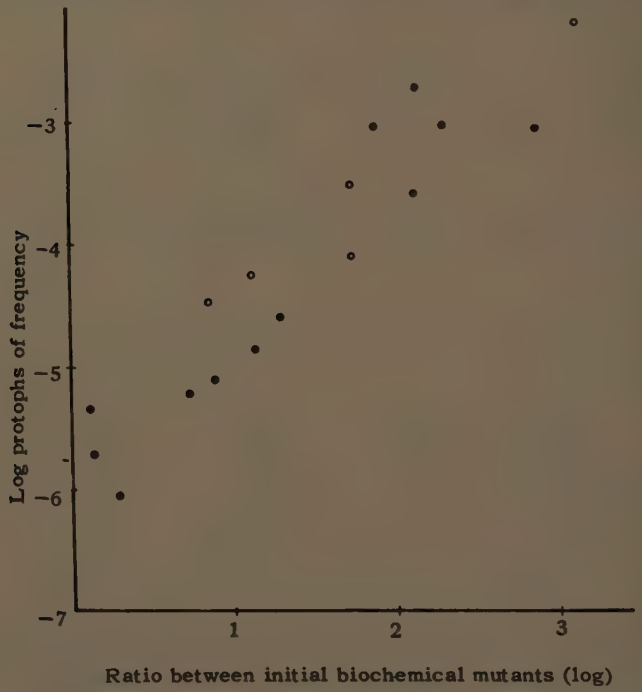

FIGURE 11. The dependence of the prototroph frequency in combination 101-870 hist × 8229-310 val + isl upon the ratio of parent forms. Key: ●, the smaller amount of spores from 101-870 hist.; ○, the smaller amount of spores from 8229-310 val + isl.

was of interest to determine whether a change in the ratio of the parent forms influences the frequency of their formation in a similar manner. The corresponding data on combination 2a-870 hist × 8229-310 val + isl are presented in TABLE 18. These data show that the increase in the frequency of formation of Type I prototrophs resulting from a change in the ratio of the parent forms is smaller than that of prototrophs belonging to Type III; because of this, the proportion of the latter is increased markedly.

The change in the proportions of different prototroph types, due to an increase in recombination frequency, was also observed in other crosses, the proportions of prototrophs with asporogenic colonies being, as a rule, increased at the expense of the decrease in the proportions of prototrophs with sporulating colonies.

Thus, it is very easy in this way to increase the frequency of prototrophic recombinants several hundred times. In this case, however, the amount of different types of these recombinants is changed greatly. The cause of the latter phenomenon is not yet clear.

Frequency of recombinations as dependent upon the time of mixed growth of the parent forms. To determine the time of maximum recombinant formation in a mixed culture of two biochemical mutants, the spores from the mixed culture were plated, beginning with the third or fourth day after plating the mixed suspension in Petri dishes.

In addition to the spores, the suspension of mycelial fragments obtained

Table 18

Frequency of Various Type Prototrophs in the Mixed Culture of Biochemical Mutants 101-870 hist and 8229-310 val + isl

Ratio 870:310	Prototroph frequency	Total number of prototrophs studied	Type I prototrophs (percentages)	Type II prototrophs (percentages)
		Experiment No. 1		
1:2	9×10^{-7}	24	25	75
1:200	9×10^{-4}	84	7.1	92.9
50:1	6×10^{-6}	67	13.4	86.6
		Experiment No. 2		
1.3:1	4.9×10^{-6}	96	38.5	61.5
1:750	6×10^{-4}	96	7.3	92.7
1400:1	5.8×10^{-3}	93	5.3	93.7
		Experiment No. 3		
2.5:1	1×10^{-6}	54	61.1	38.9
1:40	6×10^{-4}	66	4.5	95.5
		Experiment No. 4		
1:2	2.8×10^{-6}	52	30.8	69.2
1:200	4.5×10^{-4}	54	0.0	100.0

after grinding the substrate mycelium of a mixed culture of two biochemical mutants followed by filtration of the pulp through a cotton plug also was plated.

In these experiments 2 combinations of biochemical mutants were used, such as 2a-870 hist × 8229-310 val + isl and 2a-870-2 hist, val + isl × 8229-1361-1 met + thr. In both cases the results were similar.

On the plates inoculated with the spores the recombinants began to emerge on the fourth or fifth day after plating the biochemical mutant mixture. In 6 or 7 days the frequency of their emergence markedly increased and continued to increase on the following days. On the plates inoculated with the vegetative mycelial fragments the recombinants began to emerge later and were not so abundant as on the plates inoculated with the spores. The corresponding data on the crosses between 2a-870 hist and 8229-310 val + isl are presented

in TABLE 12 and on FIGURE 12. The results show that in order to obtain the greatest number of recombinations, spores from the eighth- to tenth-day mixed culture of 2 biochemical mutants should be used.

The fact that recombinants begin to emerge in the aerial mycelium earlier than in the substrate mycelium may serve as evidence of recombinant formation at the stage of sporulation. Moreover, an increase in the sporulation and in the absolute number of spores is accompanied by an increase of both the absolute number of recombinant spores and their proportion to nonrecombinant spores. This conclusion is confirmed by the absence of recombinations in most of the experiments with the cultivation of the biochemical mutants in the

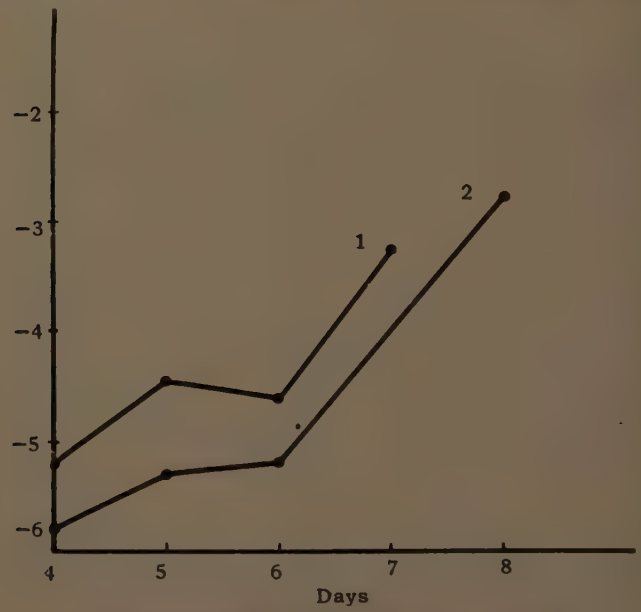

FIGURE 12. The dependence of the prototroph frequency in combination 101-870 hist × 8229-310 val + isl upon the time of mixed growth of the parent forms. Curve 1 represents experiment No. 1 (ratio of 870 to 310 is 1:100); curve 2, experiment No. 2 (ratio of 870 to 310 is 1:35).

liquid complete medium, that is, in conditions absolutely unfavorable for the formation of aerial mycelium.

DISCUSSION

Insofar as the mechanism of the genetic interaction in actinomycetes is concerned, different points of view exist. According to one of them, which is widespread, actinomycetes display not only heterokaryosis but synkaryosis, as a result of which genetic recombinations occur.[16-18] The latter are described in only four species, whereas heterokaryosis, according to Braendle and Szybalski, is much more widespread.[14]

Another opinion on this matter is held by Bradley, according to whom the so-called recombinations in actinomycetes are none other than stable hetero-

karyons containing complete genomes of both parents.[27, 28] Bradley's conclusions are based on the following observations made by him in *S. coelicolor*: (1) prototrophs segregate parent forms; and (2) supposed recombinants that are characterized by the mutant phenotype of both parents return to one or the other parent forms or become prototrophs segregating both parent forms.

Facts obtained by us in the course of studying genetic recombinations in *S. rimosus* do not permit us to make a choice between these hypotheses. On the one hand, a number of facts conflict with the concept of stable heterokaryons. The following three facts contradict it:

(1) The majority of prototrophs obtained as a result of mutual cultivation of biochemical mutants are stable and do not segregate parent forms in a number of generations.

(2) Some prototrophs frequently segregate only one of the parent forms and never segregate the other.

(3) Many auxotrophic recombinants are stable and retain their properties after a number of passages.

On the other hand, all phenomena observed cannot be explained from the point of view of the genetic recombination mechanism known for higher organisms. Special attention should be paid in this case to the fact that in all studied combinations of *S. rimosus* several types of prototrophs markedly differing in morphologic features and antibiotic activity appear. However, the majority of biochemical mutants taken as parent forms do not display any marked variability in morphologic features.

If one proceeds from the fact that the recombinants in actinomycetes are haploid ones, it is impossible to explain the fact that several types of prototroph recombinants emerge. It is the more inexplicable because the prototroph recombinants between one set of biochemical mutants do not resemble the prototroph recombinants between other biochemical mutants of the same strains and, at the same time, they all differ from the inital strains from which these biochemical mutants were obtained. These facts, however, become clear if one assumes that the prototroph recombinants in the heterozygous condition contain biochemical deficiency factors of one or both parents, for a change in the nutritional requirements of the latter is accompanied by a change in morphologic features and antibiotic properties. In case of such an assumption, various types of prototrophs in the heterozygous conditions must contain various genetic factors.

To verify this assumption, crossings between niacin-requiring mutants (63ni) and vitamin B_2-requiring mutants (58B_2) were effected. As mentioned above, the addition of niacin or vitamin B_2 to the nutrient medium greatly stimulated the antibiotic synthesis by these mutants. In analyzing the properties of prototrophs obtained for these crossings, we proceeded from a conjecture that, if these prototrophs are heterozygous with respect to one of the factors of biochemical deficiency of their parents and if partial dominance of the normal allele of this factor exists, then the activity of the prototrophs should increase with addition of niacin or vitamin B_2 to the medium. A comparative study of prototroph activity was also carried out, using corn-steep media with and without the addition of niacin or vitamin B_2. The results of this study showed that the activity of prototrophs was not greatly changed on media containing

niacin or vitamin B_2, although in some experiments an increase of their activity on media containing niacin was observed.

It should be noted that the data obtained are not sufficient to enable us to draw any definite conclusion for or against our assumption concerning the genetic structure of recombinants, for we proceeded from partial dominance of the prototrophic property which is not the usual occurrence.

When studying the antibiotic activity of recombinants, we encountered the fact that the activity of different prototroph types varies greatly. When biochemical mutants obtained from strains with different requirements in inorganic phosphorus were used for crossings, the activity of recombinants was also very different. Some recombinants, as for example, one of the initial strains, increased their activity with the increment of corn-steep liquor concentration; the activity of other strains under these conditions decreased markedly. However, any conclusions about the hereditary character of requirements in inorganic phosphorus made on the basis of these data would be premature. Indeed, the recombinants obtained from crossing biochemical mutants of one strain had the same marked differences in this respect.

The problem of inheriting the antibiotic activity in actinomycetes undoubtedly requires special and more thorough investigations. The answer to this question is connected inseparably with the solution of the problem of the mechanism of recombinations in actinomycetes.

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GENETICS OF PENICILLIN PRODUCTION

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The availability of the gene recombination process in a given microorganism shifts the resolving power of genetic analysis from the level of the nucleus to the level of the gene, or beyond. This process provides a tool for a detailed step-by-step analysis of biosynthetic pathways and for bringing together, in a new strain, properties originally scattered among different strains. Therefore, it could be of invaluable assistance in the study of biosynthesis, as well as in the breeding of strains with higher yields of useful substances. Nevertheless, for about fifteen years after the beginning of the antibiotics era very little attention was paid to the possible use of cross-breeding techniques in the study and improvement of antibiotic production. Only mutagenic treatments and selection have been applied extensively to improve the antibiotic productivity of microorganisms.

The main obstacle to the application of genetics to the antibiotic-producing microorganisms was the absence of sexual reproduction in all species used in the industrial fermentations. *Penicillium chrysogenum*, the mold-producing penicillin,[38] is a member of the fungi Imperfecti, whose sexual form is not known. Nevertheless, gene recombination has been obtained in this species[1, 2] by means of the parasexual cycle.[3] This cycle provides also a heterokaryotic phase and a stable heterozygous diploid phase, which are of considerable help in genetic studies.

Parasexual Cycle

The parasexual cycle was discovered and critically analyzed in all its phases by Pontecorvo and his collaborators[4-8] in the Ascomycete *Aspergillus nidulans*. In *A. niger*[9] and in *P. chrysogenum*[2] the parasexual cycle occurs in the absence of sexual reproduction. The successive events that ultimately lead to gene recombination are not related to the differentiation of the mycelium and are not localized in special organs as they are in the sexual cycle; they are scattered in the vegetative mycelium and occur at a very low frequency, with no apparent synchronization.

The various phases of the parasexual cycle are shown schematically in FIGURE 1. Three cycles are considered:

(1) The asexual cycle, involving a single haploid strain.

(2) The heterokaryotic cycle, including the first and involving two strains whose nuclei are brought together in the same continuous cytoplasm to form the heterokaryotic mycelium and are dissociated during the formation of the uninucleate conidia. No transfer of genetic information takes place between different nuclei in the heterokaryotic hyphae, but cytoplasmic determinants contributed by one strain may be included in conidia carrying the nucleus of the other strain and emerging from the mixed cytoplasm of the heterokaryon.

(3) The parasexual cycle, including the heterokaryotic cycle, and eventually leading to the formation of haploid recombinant strains after the establishment

of a heterozygous diploid phase. Pairs of genetically different nuclei occasionally fuse inside the heterokaryotic mycelium, as proved by the occasional detection of diploid heterozygous clones, whose single nuclei carry the complete genetic information of the two components of the heterokaryon. A heterozygous diploid clone breeds true through the uninucleate conidia, but some of the diploid nuclei undergo processes of chromosomal rearrangement, giving rise to segregant clones that exhibit recessive characters of the component strains, either in parental or in nonparental combinations. Two distinct and

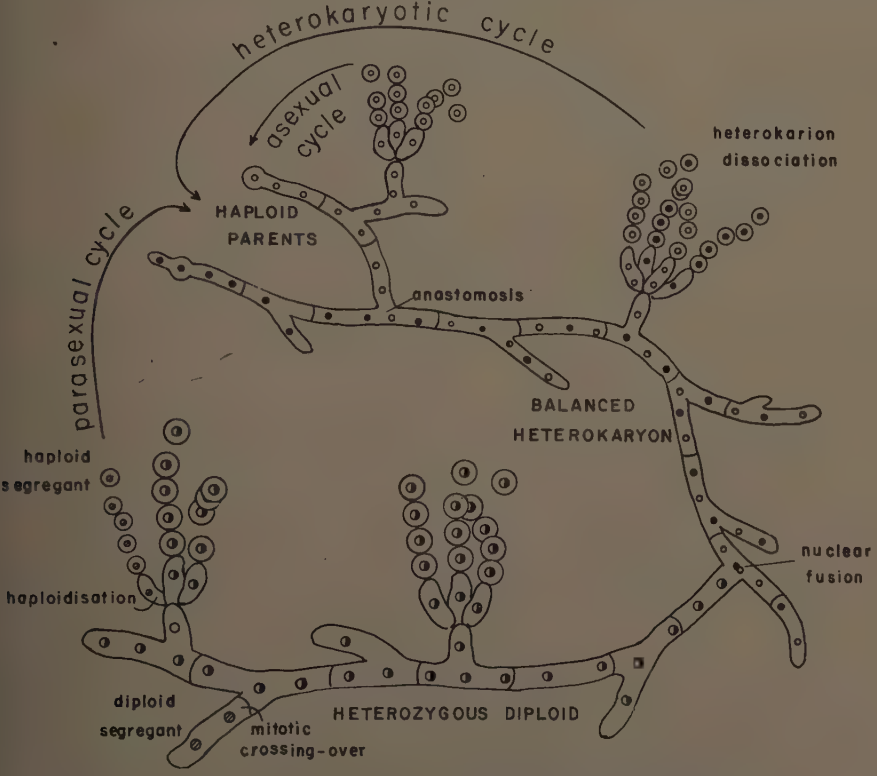

FIGURE 1. The parasexual cycle in *P. chrysogenum*; for explanations see text.

noncoordinated processes lead to the somatic segregation:[5, 10] haploidization, which eventually results in the establishment of haploid clones containing a single set of randomly assorted intact chromosomes; and somatic crossing-over, which gives rise to clones carrying diploid nuclei that are homozygous for some of the originally heterozygous markers and still heterozygous for others. The homozygosis involves markers located in a single chromosome arm, distal to the site of the crossing-over. Second-order segregation of other markers can take place later.

To study the parasexual cycle, complementary deficient strains, unable to grow on a minimal medium, commonly are used. To establish and maintain the heterokaryotic phase, hyphal growth on minimal medium is selected; to

detect heterozygous diploid clones arising from the heterokaryon, conidial growth on minimal medium is selected. Technical procedures will not be discussed in this paper and may be found in previous publications.[11-13] A diagrammatic representation of techniques used to study the parasexual cycle in *Penicillium* is given in FIGURE 2.

Somatic segregation occurs at a relatively low rate. Selective devices for the detection of segregants have been applied,[3, 11] as well as treatments that

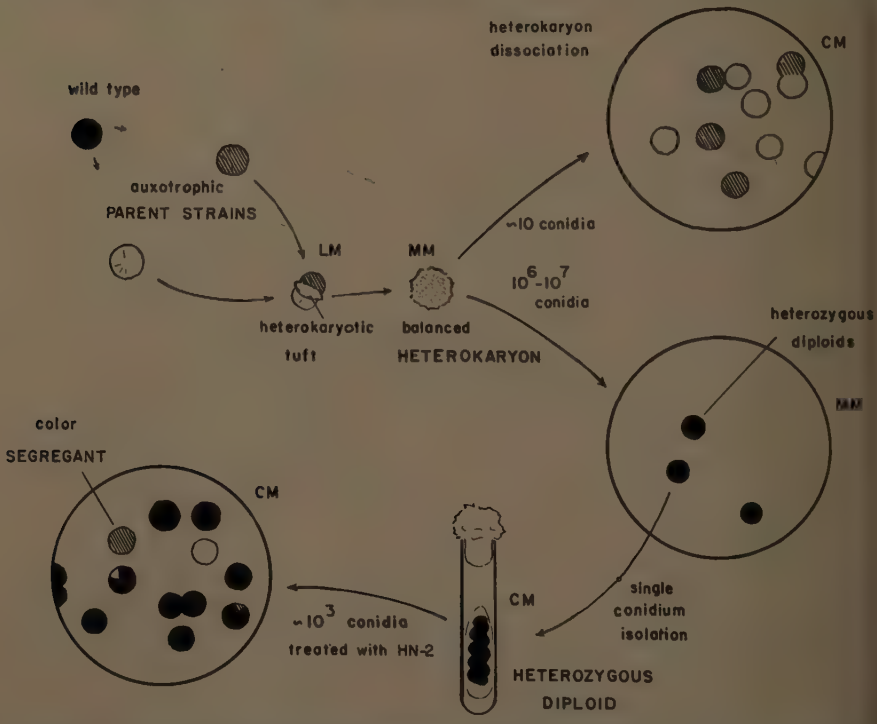

FIGURE 2. Procedures used in the parasexual cycle in *P. chrysogenum*. A heterokaryotic clone is detected on limiting medium (LM) as an overgrowing tuft in the region of contact between the two parental strains. This clone is maintained on a minimal medium (MM). The heterozygous diploid is isolated by plating conidia from the heterokaryon (10^6 to 10^7 conidia/plate) on MM. Rare colonies having the phenotype of the wild type appear on these plates, and are the heterozygous diploid. Segregants are isolated by plating diploid conidia, usually after treatment with nitrogen mustard (HN-2) on complete medium (CM).

increase the rate of segregation.[14, 15] In *P. chrysogenum* the segregation of a color marker in more than 20 per cent of the colonies deriving from heterozygous diploid conidia surviving nitrogen mustard treatment (HN-2) has been observed.[15] Among more than 500 untreated control conidia not a single segregant was scored. Although the processes that lead to the induced somatic segregation are yet unknown, it is evident that practically all of the induced segregants derive from independent events.

The parasexual cycle provides, therefore, two successive stages of nuclear integration: the heterokaryotic and the heterozygous diploid. Among segre-

gants from the latter condition, interchromosomal as well as intrachromosomal recombinants can be obtained; from the heterokaryon, strains can emerge carrying the nucleus of either parent in a new mixed cytoplasm.

All of these processes provide tools for genetic analysis and for breeding experiments in *P. chrysogenum*. Some results of such studies will be reported in the following sections.

Complementation and Dominance Relationships Between Alleles Controlling Penicillin Productivity (TABLE 1)

From a strain producing penicillin less productive (negative) or more productive (positive) mutant strains can be obtained.

The positive mutant alleles hereafter will be designated by the symbol *pp*, the negative by the symbol *np* (or *p* when they completely suppress the production).

TABLE 1

RECESSIVENESS OF NEGATIVE AND POSITIVE MUTATIONS AFFECTING PENICILLIN PRODUCTIVITY IN *PENICILLIUM CHRYSOGENUM*

Penicillin production			Genotype* of heterozygous diploids	Reference Nos.
Parental strains		Heterozygous diploid		
Standard†	standard	standard	+/+	17, 32, 33
Standard	reduced	standard	+/np	17
Standard	enhanced	standard	+/pp	32
Reduced	enhanced	standard	np +/+ pp	this paper
Reduced (1)	reduced (2)	standard	np_1 +/+ np_2	10
Enhanced (1)	enhanced (2)	standard‡	pp_1 +/+ pp_2	32

* For explanation of symbols see text.

† The production of the strain (+) from which the parental strains have been derived is used as standard.

‡ In diploid Wis. 53.844/Wis. 51.20; it was higher than the standard in the other 2 diploids.

tivity). Different subscripts will indicate different loci. The same symbols will be used to designate the phenotypic expression of the mutations. In some instances the symbols will be used to indicate, instead of single mutant alleles, groups of positive or negative mutant alleles (or the corresponding phenotype). The symbol + will designate the wild alleles.

Recessiveness of negative mutations. Among the *np* mutants, those that fail to produce any detectable amount of penicillin (*p*) are of particular interest. They are presumably defective in some biosynthetic step along the pathway leading to penicillin formation. After ultraviolet (UV) treatment they appear at a rate of about 0.4 per cent from moderately productive strains,[16, 17] but they are much rarer from very highly rated strains.[18] Heterozygous diploids between nonproducer strains and producer strains are always producers,[10, 17] the negative mutations thus appearing to be recessive (although with one mutant the recessiveness is not complete).

Complementation between negative mutations. It has been well established that the unique penicillin structure is formed by condensation of an organic acid, L-cysteine, and valine, or a close derivative of the latter.[19] Since the

synthesis of these compounds is not affected in the *p* mutants, the block should concern the conversion of these compounds into penicillin. It is not likely that such a conversion could take place in one step; intermediate compounds should be involved with different genes, therefore, controlling successive stages of the condensation. Biochemical studies[20-24] have not led to the detection of any possible penicillin precursor other than the three simple components already mentioned.

The discovery of blocked *p* mutants showing complementation would suggest the existence of a further biochemical intermediate. Bonner grew about 50

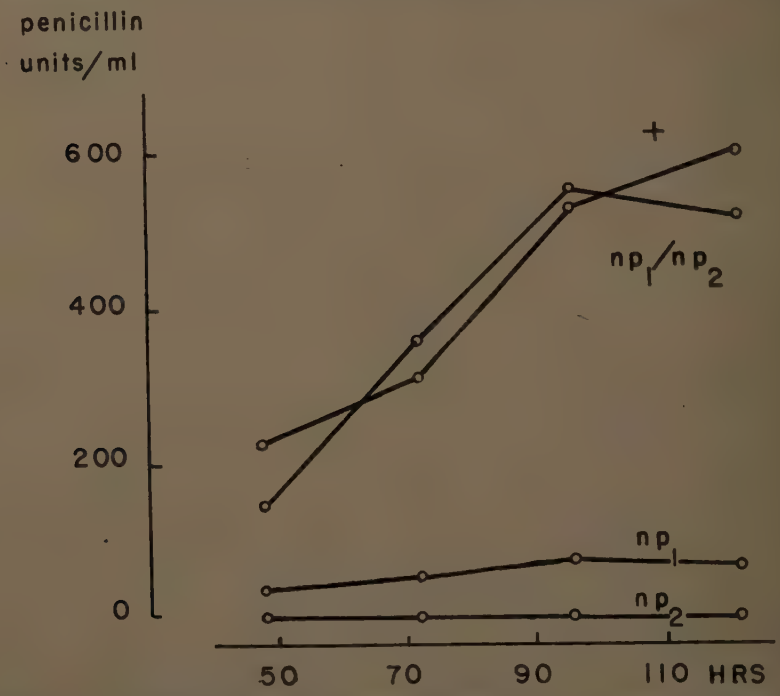

FIGURE 3. Penicillin production in submerged culture of two "negative" mutants: np_1 and np_2; their common ancestor Wis. 47. 1564 (+); and their heterozygous diploid (np_1/np_2). Medium: corn-steep lactose. Four flasks in each run.

different blocked strains in several combinations, but no complementation was found.[16] Caglioti and Sermonti[17] and Sermonti[10] have synthesized 11 heterozygous diploids, utilizing different combinations of 8 independent blocked mutants, and none of them produced penicillin. This suggests a single locus controlling the condensation of the 3 components of penicillin. Other explanations for the identity of the detected mutants can be suggested: an exceedingly high mutation rate of 1 among several loci controlling penicillin biosynthesis; antibiotic activity of some precursor of penicillin (for instance, N-phenylacetyl-L-cysteinyl-D-valine[22]), which would not have allowed a mutant producing it to be detected among "inactive" mutants; and alternative pathways to penicillin biosynthesis, except for one obligatory step which alone could suppress completely penicillin production. A mutant strain has been found[10] yielding

one tenth as much penicillin as its parent. Four heterozygous diploids between this strain and 4 completely negative derivatives of the same ancestor were fully productive, showing complementation of 2 different mutations (FIGURE 3). Complementation was observed in the heterokaryotic condition as well, but a mixed culture gave just the average yield of the component strains. Possibly a nondiffusible precursor is involved: at the present stage of the research it is not yet possible to decide.

Recessiveness of positive mutations. Mutant strains with an increased penicillin productivity (positive mutants) have been obtained in several labora-

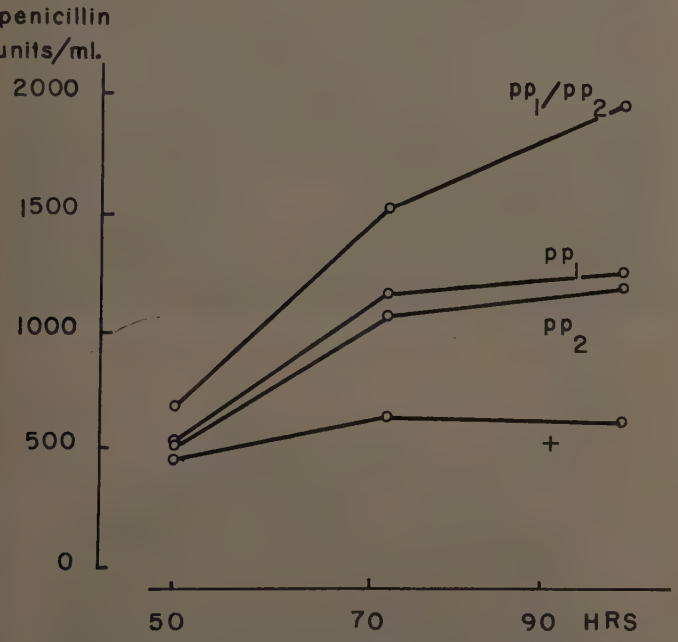

FIGURE 4. Penicillin production in submerged culture of two "positive" mutants: pp_1 (Wis. 49.133) and pp_2 (Wis. 50.1247); their common ancestor Wis. 48.701 (+) and their heterozygous diploid (pp_1/pp_2). Medium: corn-steep lactose. Three flasks in each run.

tories.[26-30] The best-known family of such mutants is the so-called Wisconsin family,[31] obtained in the Department of Botany of the University of Wisconsin, Madison, Wis. The genetic analysis of positive mutants is uncertain, both because of the poor reproducibility of the quantitative results of the fermentations[31] and because of technical difficulties in synthesizing heterokaryons and heterozygous diploids between highly rated strains, all of which grow sparsely.[32] Only a small proportion of the attempted syntheses succeed, and they often require elaborate selections whose effects cannot be predicted.

The yield of a homozygous diploid (heterozygous only for genes that do not affect penicillin productivity) is not significantly different from that of the corresponding haploid[32, 33] (FIGURE 7).

Two heterozygous diploids were synthesized between two positive mutants and their respective less productive ancestors. They behaved, as did the

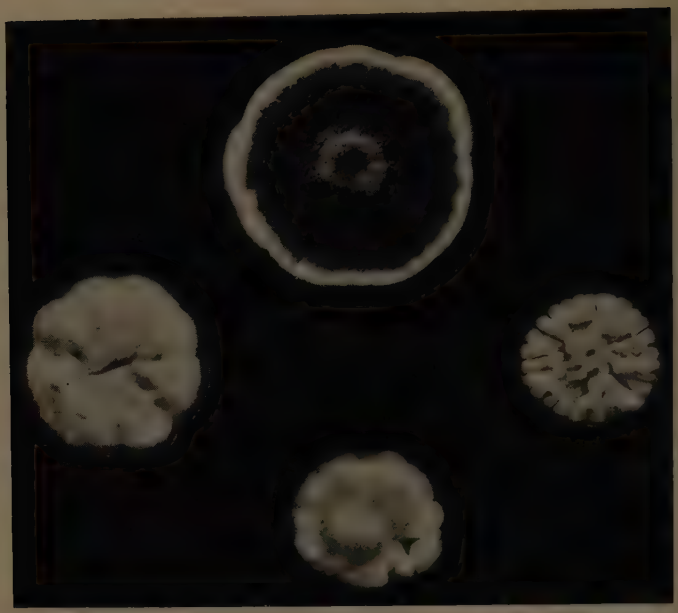

FIGURE 5. Colonies of strains: Wis. 51.20 (*left*) and Wis. 53.844 (*right*), their common ancestor Wis. 47.1554 (*top*) and their heterozygous diploid (*bottom*).

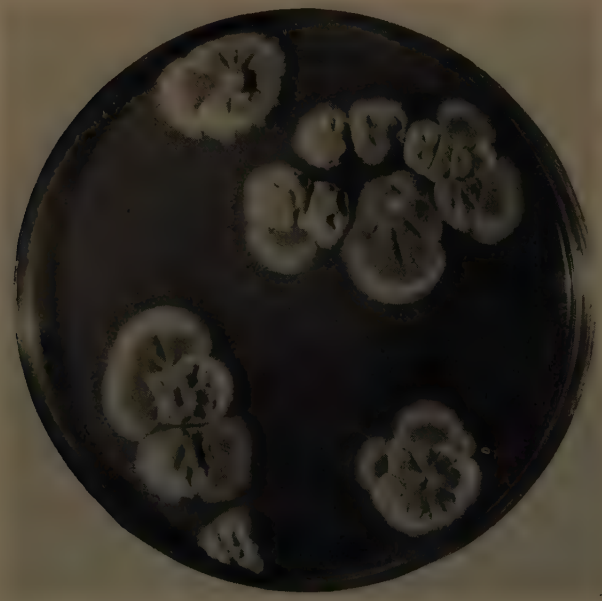

FIGURE 6. Colonies of heterozygous diploid Wis. 51.20/Wis. 53.844 growing on complete medium. Observe the emergence of overgrowing sectors, due to a cytoplasmic effect.

lower-yielding parents, in agreement with the expectation that positive mutations causing an increase in penicillin productivity are recessive (FIGURE 7).

Heterozygous diploids involving independent positive mutants. Starting from strain Wis. 47.1564, the Wisconsin family[31] has been selected along three independent lines, thus providing three series of strains carrying independent positive mutations. One of these lines, the nitrogen mustard line, is outstanding in comparison to the others, and the best strains of this line have been used to start further selections in several industrial laboratories. Strain AL 206, which has been used in some of our experiments, derives from strain Wis. 49.133 of the nitrogen mustard line of the Wisconsin family.

Three heterozygous diploids have been obtained between pairs of strains carrying independent positive mutations. Two of them were between strains

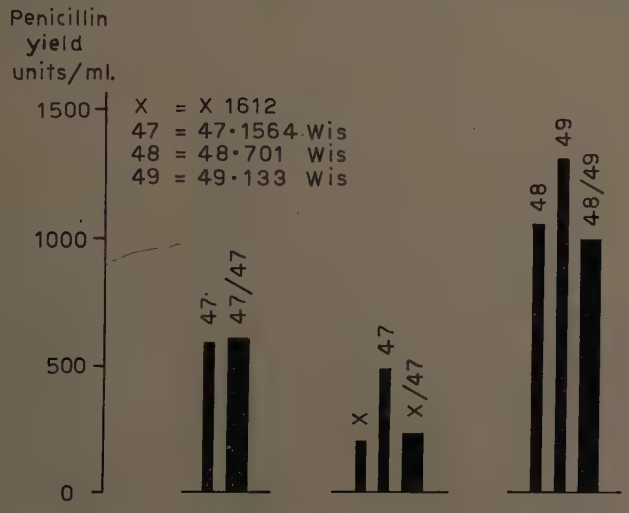

FIGURE 7. Penicillin yield of three diploids (*wider strips*) and their component haploids (*thinner strips*).

differing widely in penicillin productivity (Wis. 51.20/Wis. 53.844[32] and AL 206 Wis. 53.844), strain Wis. 53.844 being the less productive in both crosses. The heterozygous diploids gave yields inferior to or equal to that of the less productive parent (FIGURE 8); a comparison with the yield of the common ancestor, Wis. 47.1564, of the parent strains is not reliable, for this may have undergone changes during the years since the positive mutants have been derived from it. These results are consistent with the idea of recessive, nonallelic positive mutations in the haploid parents of the two heterozygous diploids, the recessiveness perhaps being not complete.

The third heterozygous diploid was synthesized between strains that gave similar yields under our fermentation conditions: Wis. 49.133 and Wis. 50.1247.[32] It consistently produced more than the component strains (FIGURES 4 and 8); nevertheless, in the Botany Department of the University of Wisconsin, where strain Wis. 49.133 produces about 50 per cent more than strain Wis. 50.1247,[31] the heterozygous diploid was surpassed by strain Wis. 49.133. In our labora-

tory, however, the heterozygous diploid has reached yields of 2100 U./ml., which is beyond the range of productivity of strain Wis. 49.133, both in our Institute and at the University of Wisconsin. The actual superiority exhibited in our laboratory by the diploid Wis. 49.133/Wis. 50.1247 in comparison with the component haploids is difficult to interpret on the basis of nonallelic recessive positive mutations in the 2 parent strains.

A heterozygous diploid between a completely negative mutant and a positive mutant (AL 206) from a common ancestor (Wis. 47.1564) gave a production similar to that of the common ancestor.

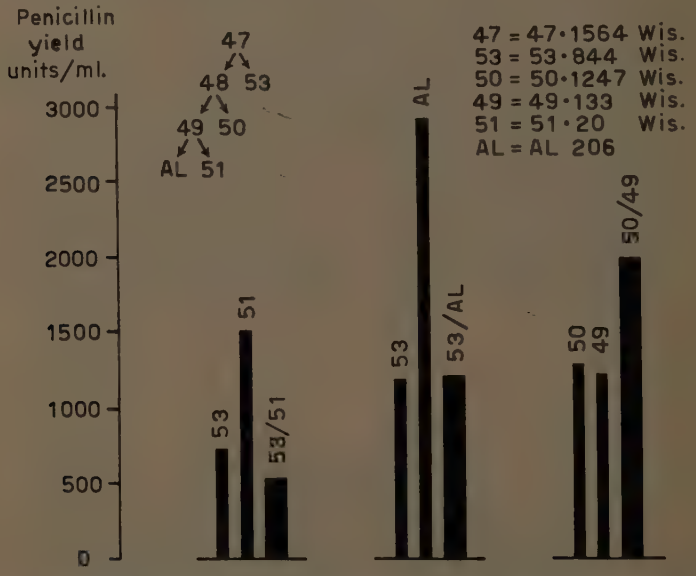

FIGURE 8. Penicillin yield of three heterozygous diploids (*wider strips*) and their component haploids (*thinner strips*). At the top left the genealogical tree of the haploid strains is indicated.

Dissociation of Heterokaryons: Lack of Autonomous Cytoplasmic Control of Positive Mutations

A heterokaryon[34] has been synthesized between the wild strain NRRL 1951 and a remote positive derivative of it, strain Wis. 49.133. The latter differs from its wild ancestor in having a much higher penicillin productivity and in its dwarf habit of growth. The first strain has been marked with a proline requirement, the second with a pyridoxine requirement. Single heterokaryotic hyphae were isolated and, from the conidia of the derived colonies, prolineless and pyridoxineless dissociates have been obtained. They were all dwarf, notwithstanding the nucleus they carried. If no transfer of genetic information is assumed to take place among nuclei of the heterokaryon, the dwarf dissociates carrying the wild nucleus must differ from the original wild strain in some autonomous cytoplasmic component. The cytoplasmic dwarfs are not stable and regularly sector, producing wild phenotypes. Cytoplasmic dwarfs, revertant wild types, and the original wild type do not differ significantly in penicillin

productivity; the productivity of the pyridoxineless dissociates from the heterokaryon equals that of the pyridoxineless Wis. 49.133 parent. Thus, the penicillin productivity shows a complete association with the nuclear markers and no relation with the cytoplasmic composition affecting the growth habit.

It may be concluded that the positive mutations that have been accumulated in strain Wis. 49.133 during its selection from the wild type are strictly nuclear, that is, gene-controlled. These mutations have been selected in at least 9 successive steps, following X irradiation, UV irradiation, nitrogen mustard treatment, or no mutagenic treatment.[31]

A heterozygous diploid[35] has been synthesized between 2 independent positive mutants (Wis. 51.20/Wis. 53.844), both exhibiting dwarf habit of growth and both derived from strain Wis. 47.1564. The diploid was dwarf (FIGURE 5) but unstable, reverting through sectors to the normal-growing phenotype of strain Wis. 47.1564, probably due to some cytoplasmic restoration (FIGURE 6). No significant change in penicillin productivity paralleled this cytoplasmic reversion.

Segregation of Heterozygous Diploids

Genic control of negative mutations. From a heterozygous diploid (penicillin producer), synthesized between a producer strain and one of its negative mutants, producer and negative segregants have been obtained. The *p* locus shows linkage with several markers[13, 17] and appears to be distal to a yellow mutation on the same chromosome arm.

From a heterozygous diploid (producer), synthesized between a *p* and *np* mutant, both completely and incompletely negative segregants have been obtained, as well as some producer haploid segregants, indicating that the two negative mutations are located on nonhomologous chromosomes.[10]

Segregation of heterozygous diploids involving independent positive mutations. The segregation of positive mutations from a heterozygous diploid may be studied by following two main lines. Segregation of suitable markers not connected with penicillin productivity can be detected and the segregants can be tested for productivity and ploidy, or derivatives from the heterozygous diploid can be screened directly for penicillin productivity. Stimulation of segregation by mutagenic treatment is an essential preliminary in these experiments. The first approach is more efficient, for only strains that are obviously the result of some segregation process are tested for penicillin productivity. It is, however, more restrictive since only a portion of the possible products of segregation is detected unless numerous markers are introduced into the heterozygous diploid (which is an undesirable condition, since most of the nutritional or color mutations affect, somehow, penicillin productivity).

Segregation from the heterozygous diploid AL 206/Wis. 53.844 has been explored along both lines (TABLE 2). The AL 206 parent was marked with a proline requirement (code No. 207 *pr*, yield 3058 ± 21 U./ml.) and the Wis. 53.844 parent with a nicotinamide requirement (code No. 181 *nic*, yield 1270 ± 45 U./ml.). The heterozygous diploid was prototrophic and produced 1308 ± 65 U./ml. (average yield of three clones). The common ancestor of the component strains produces between 400 and 700 U./ml.

Fifty auxotrophic segregants have been recovered after HN-2 treatment of conidia of diploid AL 206/Wis. 50.1247. Forty-nine were prolineless and one

TABLE 2

FREQUENCY DISTRIBUTIONS OF PENICILLIN PRODUCTIVITY BY HETEROZYGOUS DIPLOID AL 206/WIS. 53.899 (207 *pr*/181 *nic*), HAPLOID PARENTAL STRAINS, FIRST-ORDER AND SECOND-ORDER SEGREGANTS

																			X V	U M A	Y T Q	W P I E	S D	O C	Z H	N L G	R	B	Control cultures (strain AL 206)
														W		W W		W	W										Second-order prolineless segregants (HN-2) from diploid N*
	T	V U	T	T	T	U T	Z Z T	U U U U	U U	Z	Z Z T T U	Z U	T	U	U	U U	Z U	U T			Z T T	T T	Z T T		T T				Second-order prototrophic segregants (HN-2) from diploid N*
L L	L*										"L"		L	L	L L L	L L L L	L L L L L L L	L L L L L L L	L L L	I I I I L L	I I I I I L L L	I I L	I I	L					First-order auxotrophic segregants† (HN-2) from diploid 207 *pr*/181 *nic*
									X X X X X X								X S	Q Q Q P P P	Q Q P P P P P P O O	S S	S S S S	P P N N N M X S S							First-order prototrophic segregants (HN-2) from diploid 207 *pr*/181 *nic*

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
	S H H	Q H		X	Q	O	X Q	X X X X S Q Q	X Q Q Q Q P M H	X X X X X S Q Q Q Q P	X X X Q Q Q Q O O O P H	S S Q Q Q P P O M M M M M M M	X S Q Q O O O H H	X X X X S Q P P O O O M H H H	X X X Q O M M H	X X X X X P O O M H	X S Q P P O M M H	P P M	X X O O P M M M	Q Q P P O O M M M M M M M H H	O O O O O O M M M M M H H H H	O O O O O M M M M M M H H H H	S Q Q Q Q P P O O M M H H H H	S Q O O O P P M M M H H H H H	S S S S S P P P O O M M M M H	S P P P M M	S S S P N M	S S S Q* P O		N*	N N					
Heterozygous diploid 207 *pr*/181 *nic*										H	B B A A A	B	H B B A		B B	B	B																			
Haploid parental strains						Strain 181 *nic*			A	C A	G CC		B B	G F F B						Strain	207		*pr*		A	E A	E E	E E E	E E D D C	E E B	E C B B	B B	G		G F	F
(X 100)	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35

* Segregant clone classified as diploid.

† All of these strains were prolineless with the exception of "L," which was nicotinamideless.

Each capital letter represents a single shake-flask culture (corn-steep lactose medium), with the exception of the "control cultures" which represent pooled groups of two or more cultures. Identical letters indicate flasks of the same run. The penicillin production of each culture may be read on the bottom line of the table, where 1 unit represents 100 units of penicillin per ml. For the first- or second-order segregants each letter refers to a different clone. A single clone of each of the two parental strains and of the control strain AL 206 was tested in several flasks and runs. Three clones of the heterozygous diploid were repeatedly tested.

was nicotinamideless. All of them were haploid, except for one prolineless segregant, which produced only 100 U. of penicillin/ml. The haploid prolineless segregants formed a compact group with respect to penicillin productivity, which ranged between the average yield of the heterozygous diploid and that of the prolineless parent. There was, therefore, a pronounced increase in penicillin productivity connected with the segregation of the chromosome carrying the prolineless marker, but it never led to yields as high as those of the prolineless parent. The nicotinamideless segregant behaved as the nicotinamideless parent.

Two hundred and seventy-eight prototrophic colonies, also derived from conidia of diploid AL 206/Wis. 50.1247 surviving HN-2 treatment, were tested directly for their productivity. They fell into 2 clearly separated groups. The average yield of the less productive group was similar to that of the heterozygous diploid, while that of the more productive group approached the average yield of strain Al-206. The strains of the second group were evidently all segregants, and they amounted to more than one half of the total tested. The 27 most productive strains were tested for ploidy (conidial size) and all were haploid except 2, indicated as N* and Q* from the code letter of the corresponding run. The occurrence of highly rated haploid prototrophic strains indicates the presence of positive mutations on chromosome(s) differing from those carrying the prolineless and the nicotinamideless markers.

These results point to a distribution of genes affecting penicillin productivity on different chromosomes, but as yet do not give evidence of any cumulative effect of independent positive mutations.

Second-order (HN-2-induced) segregation has been studied from the first-order segregant diploid N*. Five prolineless segregants gave yields similar to those of the first-order prolineless segregants, and inferior to that of diploid N*. The productivity of 43 prototrophic subclones was widely scattered, ranging from that of the N* diploid down to several hundred units of penicillin per milliliter. The distribution resembled closely that of the productivity of the first-order prototrophic segregants. The decline of productivity of most of the second-order segregants suggests the presence of recessive negative mutations in the heterozygous diploid N*.

A similar decline of productivity was observed in many first-order segregants from diploid Wis. 49.133/Wis. 50.1247 (*see above*).[32]

The Practical Utilization of the Parasexual Cycle

Pontecorvo[3] has suggested that the rational use of the parasexual cycle in microorganisms may permit one "to introduce into the breeding of improved strains procedures similar to those used by the plant breeder in the improvement of vegetables, crop plants, and fruit trees." I shall consider here some of the properties peculiar to the parasexual cycle and, in turn, compare its possible effective use in a breeding program with imperfect fungi with the well-known possibilities that exist in organisms having a sexual cycle.

In fungi having a perfect stage, the products of meiosis can be detected readily, as they are formed in a characteristic structure. Spores that are formed after the meiotic division of a heterozygous diploid nucleus carry nuclei in which

homologous chromosomes of the two parental strains have exchanged segments through crossing-over; the nonhomologous chromosomes have been assorted randomly through reductional division. In a heterozygous diploid mold that undergoes somatic segregation, however, only rare conidia will carry nuclei resulting from infrequent chromosomal rearrangements; these rare conidia will be present among a large population of the normal heterozygous diploid conidia. Thus, in the parasexual cycle recombinant nuclei are rare and adequate tests must be provided to permit their recognition. This handicap of mitotic segregation may be overcome in part. The rate of chromosomal rearrangement may be enhanced enormously by treatment with a mutagenic agent.[14, 15] The detection of clones carrying segregant nuclei may be made far more effective by the use of appropriate markers and selective devices.[5, 36]

In sexual organisms the transfer of desirable genes from one strain to another, in general, is facilitated by means of a back-cross, that is, a recombinant strain crossed with either one of its parents. This method allows a stepwise transfer of genes and so reduces the number of offspring to be tested when multiple recombinants are being sought. A somatic segregant formed after mitotic crossing-over carries one cross-over chromosome together with a parental homologous chromosome; the remaining chromosomes are present as heterozygous pairs. Such a segregant thus is similar to a back-cross in which a recombinant has been crossed with its least related parent. This is a useful aspect of the parasexual cycle, since the synthesis of the heterozygous diploid is laborious. The mitotic distribution of the chromosomes after crossing-over provides an almost automatic back-cross procedure. On the other hand, the regular partnership of the cross-over chromosomes with homologous parental chromosomes in mitotic segregants provides a serious obstacle in the rational use of mitotic segregation. That this is true is due to the fact that recessive alleles that were not expressed in the segregant diploid clones cannot be followed phenotypically. The loss of recessive useful alleles in a heterozygous diploid may occur continuously and yet remain undetected. This loss of useful recessive genes may be overcome in part when haploid recombinants are selected directly from recently synthesized diploids. Under these circumstances, however, the resolving power of the genetic analysis is limited to the level of interchromosomal recombination.

In general, the diploid phase in fungi is transient. The use of fungi, however, in which a parasexual cycle can be demonstrated means that at the same time one can select and maintain stable heterozygous diploid strains. This fact is of paramount importance, and it may well be that it is this aspect of the parasexual cycle that will prove of greatest value in practical terms in the years ahead. The heterozygous diploid may be useful for any one of many reasons. It is particularly useful when harmful recessive mutations are carried by one or both parental strains. The masking of harmful alleles in the heterozygous condition may lead to so-called hybrid vigor or heterosis. The possibility that a superior strain may be affected by harmful mutations cannot be overemphasized. Such mutations trace back to diverse sources. They may result from a change in fermentation conditions, and do result in an altered phenotypic expression of some favorable mutation. Such an altered expression also may

result from changes in the genetic background due to the induction of other mutations and, finally, undesirable mutations may accumulate during the maintenance of the stocks.

Clear evidence of the shift of positive genes to negative genes due to a change in the fermentation environment has been reported by Johnson.[37] The Wisconsin strain 47-1564 selected from the Wisconsin strain Q-176 gives a penicillin yield 1.49 higher than its parent when tested under the routine conditions in the Department of Botany of the University of Wisconsin. In the Department of Biochemistry of the same university, however, under slightly different conditions, its production was only 69 per cent of that of the parental strain. Strain Wis. 49.133, derived from Wis. 47-1564, is superior to the parental strain Wis. Q-176 even in the Department of Biochemistry, but obviously under these conditions it is affected by some negative mutations.

The second-order segregation of many less productive strains from a highly productive diploid (first-order segregant N* from diploid AL 206/Wis. 50.1247) also suggests the occurrence of negative genes affecting penicillin yield. The "heterosis" of diploid Wis. 49.133/Wis. 50.1247 also indicates the occurrence of negative mutations. To account for the superiority of this diploid in contrast to both haploid parents, which in turn are both superior to their common ancestors, the occurrence of negative mutations concealed in the heterozygous condition, however, is not sufficient. The occurrence of positive mutations also must be assumed. The phenotypic expression of such genes in the heterozygous diploid can be attributed to allelism of some recessive positive mutations in the two parental strains, to dominance of some positive mutant allele, or to the expression of some recessive positive mutant allele due to somatic segregation preceding the isolation of the heterozygous diploid strain.

As mentioned earlier, utilization of hybrid-vigor may prove to be the most important application of the parasexual cycle. The heterozygous diploid lends stability to strains and hybrid-vigor lends productivity. Screening of somatic segregants should be an auxiliary procedure in any case, both to prevent the rundown of the heterotic strains and, in turn, to pick up possible superior segregants. When heterosis is not manifested, screening of somatic segregants is, of course, indispensable.

The dosage effect of genes affecting penicillin productivity in the homozygous diploid or polyploid strains has not been detected in the initial tests. Many reasons could account for this and should be the object of far more careful investigations. The superiority of diploid, triploid, and tetraploid strains in the production of protease has been shown by Ikeda *et al.*[25] in *A. sojae.*

In concluding, it should be emphasized that the breeding procedures permitted by the parasexual cycle may not prove effective immediately in terms of practical results. At this juncture it is still too early to predict with assurance, but it may be said with confidence that such studies will contribute richly to our knowledge of antibiotic biosynthesis and, perhaps even more significantly to our understanding of the nature of the genetic changes brought about during the selection of improved strains, and thus in time should permit a rational approach to a problem that is still at an empirical level.

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Discussion of the Paper

E. Dulaney (*Merck Sharp & Dohme Research Laboratories, Rahway, N. J.*): Papers in the first part of this monograph concern theoretical aspects of *Streptomyces* genetics. The presentations of S. I. Alikhanian and of G. Sermonti treat more directly with genetic aspects of organisms producing two important groups of antibiotics: the tetracyclines and the penicillins. My remarks will be directed primarily to the industrial implications of the former paper.

Although the report of Alikhanian is concerned with tetracycline-producing *Streptomyces*, he and his associates have published a number of papers in Russian concerned with induced mutation and strain selection in other antibiotic-producing organisms.[1-5] The problems they have met are comparable to those encountered in our laboratories and, I am sure, in other laboratories in the United States as well. They need not be discussed in detail here.

The use of induced mutation followed by selection and testing of cultures, empirical as it may be, is one of the most successful and important parts of the fermentation industry.[6-7] Alikhanian and his associates have used this approach with success.[3] The Terramycin-broth potency was doubled through

a three-stage mutation and selection, and that of Aureomycin was tripled through a five-stage mutation and selection as noted in the previous paper. It would be interesting to know the number of cultures tested at each stage before a superior strain was obtained.

In addition to the superior tetracycline-producing strains reported here, success in obtaining superior albomycin producers has been achieved.[3] Albomycin is apparently the same as the antibiotic grisein.[8] Penicillin- and streptomycin-broth potencies also have been increased markedly through the use of induced mutation and strain selection. Penicillin-broth potencies in excess of 5000 U./ml. and streptomycin-broth potencies of 6000 U./ml. have been reported by Alikhanian.[3] The cultures producing these high streptomycin activities were unstable, however, and the stable strains obtained from them produced 4500 U./ml. To my knowledge these are the highest penicillin- and streptomycin-broth potencies reported in the scientific literature. Industrial firms in this country and other countries do not disclose the productivity of their cultures. It is quite clear, however, that Alikhanian and his associates have been successful in increasing yields of antibiotics through mutation and strain selection. Perhaps this success justifies the following quotation: "It seems to us that the level of activities in antibiotic-producing strains of today will seem small tomorrow." [3] How long the past and present success can be extrapolated into the future is not clear to me. However, I am as optimistic in this respect as Alikhanian is and I am sure that other contributors to this publication share this feeling. I am therefore convinced that the mutation and selection technique will be with us in the fermentation industry for some time to come. The success attained with this approach is reason enough for this statement. I am impressed by the elegant work described by G. Sermonti on the genetics of penicillin-producing cultures, as well as with the research on *Streptomyces* reported elsewhere in this monograph. The industrial implications of these studies are numerous. Even when much more is known about the genetic systems of the organisms involved, so that more direct applications are possible, I believe that such research will complement, not replace, the mutation and selection technique.

The data in Alikhanian's Figure 5, showing the correlation between morphologic variants and loss of antibiotic productivity, warrant comment. Approximately 30 per cent of these morphologic variants produce no Terramycin, and almost the same per cent produce only a slight amount. Thus, 60 per cent of the morphologic variants are essentially nonproducers. This close correlation is somewhat surprising to me, but perhaps others more familiar with tetracycline producers could discuss this more factually. The correlation could indicate a number of things, but one attractive possibility is a close correlation between morphologic change and auxotrophy. It might be expected that such a correlation might be expected, for morphologic change is merely an overt symptom of physiological change. The point is whether the loss in productivity can be correlated with auxotrophy. I have not found such a discussion by Alikhanian in his publication on auxotrophy in *Streptomyces rimosus*.[9] However, he has published some data that perhaps are pertinent.[9] Methionineless and nicotinic acidless mutants developed a dry weight equal to that of the parent strain, but produced much less Terramycin in the following medium:

corn-steep liquor, 0.1 per cent; starch, 3 per cent; $(NH_4)_2SO_4$, 0.5 per cent; NaCl, 0.5 per cent; $CaCO_3$, 0.5 per cent; and H_2O, to volume. Addition of methionine and nicotinic acid resulted in no increase in cell weight, but raised antibiotic titers to the level of the parent strain.

In addition to the mutation and selection approach, Alikhanian and his associates also have been studying possible parasexuality in microorganisms.[3, 10-12] They have reported success in increasing yields by the use of recombinants. For example, a recombinant from two auxotrophic oxytetracycline producers was reported to produce more oxytetracycline than either of the two original strains.[10] However, the productivity of the best recombinant was only 13 to 14 per cent above the productivity of the better parent culture.

A superior penicillin-producing recombinant was also claimed.[3] Alikhanian reports a new hybrid from Wisconsin-51-20 and strain G-31. His use of the term hybrid indicates to me some form of recombinant and not a heterokaryon.

Alikhanian also feels that his data indicate another possible use of recombination in microorganisms. He reports that, with successive mutation and selection of strains, some type of saturation results, so that further mutation is not productive.[3] Certainly, the excellent studies of Newcombe[13] show a genuine saturation or impairment in *Streptomyces* spores subjected to certain dosages of X rays and ultraviolet light. Thus, higher dosages of X rays and ultraviolet light did not result in higher mutation frequencies. A nuclear division did not bring a release from this saturation. It is unlikely, however, that this saturation continues after a number of transfers of the culture. Perhaps Alikhanian is not referring to this type of saturation, but to the possibility that there is a limited number of sites for favorable mutation resulting in increased productivity. At any rate, he feels that this difficulty might be overcome through the introduction, by means of recombination, of nuclear elements unaffected by irradiation. It is difficult to see how this would accomplish the aim. Replacing nuclear elements containing favorable mutations by those with no such mutations would appear to decrease productivity. Obviously, the exchange of nuclear material can cause numerous effects, both favorable and unfavorable, with regard to biosynthesis. It is not necessary to expound on the possibilities here.

Finally, I shall discuss briefly another application of the mutation technique in the fermentation industry. I refer to the use of mutagens to make strains that are capable of synthesizing new products, or antibiotics. This technique merits considerable emphasis. I do not refer to the mutation of a nonantibiotic producer to obtain antibiotic-producing strains or to the mutation of an inferior antibiotic producer to obtain strains with workable levels of productivity. While these approaches offer possibilities of success, inherent difficulties must be solved before success with them could be regarded as probable. However, mutating a culture that makes a good antibiotic has extremely interesting and industrially important possibilities. The natural occurrence of analogues of antibiotics, for example, tetracyclines, penicillins, and kanamycins,[14] shows the diverse synthetic capacities of the organisms making them. Of course, mutation and selection offer a means of obtaining strains producing a single desired antibiotic of a series.[15] More interesting, however, is the possibility of using mutagens to produce strains that synthesize new analogues. The

tetracycline producers were an obvious group for such a study. The tetracycline molecule offers numerous functional groups for alteration. Changes in the antibiotic can occur and activity be maintained, as exemplified by chlortetracycline, oxytetracycline, and tetracycline.

Recent publications by McCormick and his co-workers[16, 17] report success with this approach. They reveal the production of dimethyltetracyclines and 7-chloro-5α(11α)-dehydrotetracycline by mutants of *Streptomyces aureofaciens*. I wonder if Alikhanian has noted any new tetracyclines produced by his mutants.

The experiment discussed by Sermonti on isolation of a mutant that produces a penicillin-containing adipic acid is quite interesting and bears somewhat on this point. However, the parent of the mutant also was reported to incorporate adipic acid into the molecule. Thus, the mutant appears to be a more efficient utilizer of adipic acid as a precursor. The extension of these studies is obvious.

Success with these approaches should result in their use with other antibiotic producers.

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T. C. Nelson (*Eli Lilly and Co., Indianapolis, Ind.*): The development of the antibiotic industry has depended on empirical methods to maintain production and to discover and to increase the yield of new antibiotics. These methods are approaching the point of diminishing returns. A quantitative measure of this loss of efficiency is the frequency with which the same antibiotic is encountered in screening new actinomycete isolates and in the increasing ratio of isolates tested to new antibiotics found. The industry could take advantage of the body of knowledge of genetic interactions and methods of determining pathways of biosynthesis that have accumulated in the past decade. The papers of S. I. Alikhanian and of G. Sermonti are among the first to start closing the gap between basic and applied research. Some of the problems that the worker encounters in this intermediate zone of investigation are the subject of this discussion.

The application of the recent advances in understanding the genetics of microorganisms used in antibiotic production may be divided conveniently into two approaches. These are quantitative, increasing the yield of an antibiotic, and qualitative, modifying the chemical structure of the antibiotic. The first goal has more immediate value to development workers, but the second approach may yield larger long-term advances.

The production of strains with higher yields of antibiotic may be divided into three stages: treatment, selection, and evaluation. We have available an extensive literature detailing methods of producing the maximum amount of genetic variation by induced mutation in a population, as well as obtaining maximum recovery. Unfortunately, the results of basic research on variation give no hints, but even discourage attempts to produce specific desired modifications. Selection of desirable variants is still more an art than a science. A rapid method of screening individual members of a large population of treated microorganisms is needed as a preliminary step in evaluation. The size of inhibition zones produced in a lawn of antibiotic-sensitive bacteria seeded over actinomycete colonies can be misleading.

Evaluation is the rate-limiting step in the development laboratory. Time and economics limit the ideal of testing all possible selections in all possible combinations of growth conditions and media. Laboratory-scale methods of determining the maximum yield of an antibiotic can lead to a false conclusion concerning the level of production of an isolate. The present methods used in industry mimic, as closely as possible, the method used in production equipment, although the ratio of media volumes ranges from 100,000 to more than 1,000,000. The same cultures, media, inoculation volume ratios, intermediate steps in growth, time, and temperature of fermentation may be used at these extremes with almost the same yields of antibiotic per unit volume. However, repetitive testing of the same isolate in the laboratory often results in a wide fluctuation in yield from run to run. The source of this variation probably lies in the use of temporal rather than biological time, as measured by the stage of growth of the culture, in laboratory testing. Thus, any genetic investigation of increased or decreased yields must be free of these fluctuations in order to be significant.

A double mutagenic treatment, as used by Alikhanian, with ultraviolet irradiation to interrupt the course of DNA synthesis and an alkylating agent to supply

modified nucleic acid precursors, is an example of the application of the results of basic research to industrial development. A different approach is the selection of an isolate producing neither more nor less antibiotic, but with a pattern of growth changed in every respect other than morphologic variation. The recovery by Alikhanian of a strain requiring higher levels of phosphate is an example of such a strain. Hockenhull's hypothesis of streptomycin biosynthesis involves recycling phosphate. Biosynthesis of the tetracyclines similarly may involve constant use and regeneration of inorganic phosphate as phosphorylated intermediates are condensed to the final antibiotic molecule. In a complex medium of the type used in production the initial soluble phosphorus may be only one fourth of the total amount, the remainder being bound in the insoluble portions of components such as corn steep and yeast. At the end of the antibiotic production period one third of the initial soluble phosphorus is still present in the medium. These measurements are complicated by run-to-run variation, lysis of the mycelium in the late period of production, and leaching of phosphates from the insoluble medium components. As yet no strain has been recovered with a significantly different pattern of phosphate utilization, as measured by the final phosphate level. Strains having a pattern of sugar utilization consistently different from the parent have been isolated. The final antibiotic yield was unaffected, however.

If the biochemical pathways of antibiotic synthesis could be changed qualitatively, desirable chemical modifications might be obtained. At this time this approach is hampered by a lack of knowledge of the specific chemical modifications that are clinically desirable and, were these known, by the biological method of producing them, assuming direct chemical modification to be unavailable. A beginning has been made, either by chemical modification of the purified antibiotic (such as the propionyl derivative of erythromycin), by feeding precursors (phenoxyacetic acid to force production of penicillin V), or by screening known antibiotic-producing strains against antibiotic-resistant, dependent, or physiologically variant bacteria deficient in terminal oxidative pathways, such as those used in the Union of Soviet Socialist Republics.

An extension of this approach is the production of recombinants between actinomycete strains producing antibiotics belonging to the same chemical family or containing the same chemical moiety. An attempt at modification has been made with strains producing antibiotics containing inositol amines. The extensive series of treatments and isolations necessary to obtain auxotrophs for detecting recombination usually have resulted in a complete loss of ability to produce the antibiotic. This may not be an undesirable result if the strain retains the ability to produce several components of the antibiotic molecule.

Kenneth B. Raper (*University of Wisconsin, Madison, Wis.*): Intensive study of penicillin fermentation during World War II led to the early discovery that the capacity to produce this drug characterized virtually all isolates of the *Penicillium chrysogenum* series, of which Fleming's strain of *B. notatum* was representative. Yields varied greatly, however and, in the search for more productive cultures, one often heard expressed the almost despairing wish: "If only these penicillia had a sexual stage; if only more productive strains could be bred by applying the techniques used so successfully in higher plants by geneticists." Emphasis, of course, was placed in a different direction,

namely, that of mutating the best-producing culture then available. The success in this endeavor, initiated in my laboratory in Peoria, Ill., and brought to fruition by M. Demerec in Cold Spring Harbor, N. Y., and especially by M. P. Backus and J. F. Stauffer at the University of Wisconsin, was so dramatic as eirtually to exclude for more than a decade other approaches that in the long term may prove even more rewarding. The net result was to mutate and select, remutate and reselect with steady improvement in yield to the point where it is doubtful if penicillin is now produced anywhere in the world by a culture whose ancestry can not be traced in an unbroken line leading back through Wis.-Q176 and NRRL 1951 to the moldy cantaloupe in Peoria.

The phenomenon of heterokaryosis in asexual fungi was, of course, known from the work of B. O. Dodge, A. H. R. Buller, and many others, and in 1945 Lindgren and Andrews reported some attempts to utilize such cytoplasmic hybrids for enhancing penicillin production. The results were disappointing, and they failed to record any evidence that could provide clues to the interstrain balance achieved in their production bottles. In other studies, chiefly with citric acid-producing aspergilli, attempts to increase productivity likewise have failed (Yuill, 1953; Ciegler and Raper, 1957), in substantial part because, even though one begins with a heterokaryon, the growth resulting in the production culture, in the main, will represent a mixture of the two parent types, generally in unpredictable proportions. Using selected mutants and tailor-made media, balanced heterokaryons can be achieved in the laboratory, but the substrates required for substantial productivity seldom, if ever, duplicate those on which the balanced heterokaryon is maintained; hence persistence of the heterokaryotic state is not assured in the ensuing fermentation. Even so, the outlook is not a dismal one.

Working initially with heterokaryons in *P. notatum*, *Aspergillus nidulans*, and *A. niger*, Pontecorvo and his associates (1947, 1953) and Roper (1952) demonstrated an unsuspected alternative to true sexuality in filamentous fungi which G. Sermonti now has applied to *P. chrysogenum* with very bright promise for the future. I refer, of course, to the occasional heterozygous diploids that arise from heterokaryons via parasexuality and contain within single nuclei characters drawn from two contrasting parents. Results to date have not revealed the hoped-for over-all increases in antibiotic production, but they do provide a rational approach for the further exploitation of this possibility. Of perhaps greater significance, they have demonstrated that heterozygous diploids may be employed for the biosynthesis of new or modified penicillins possessing differing antibacterial activity by the selection of strains to be recombined and by careful attention to the substrates upon which such diploids of parasexual origin are grown.

In the breeding of higher plants, the geneticist usually has at his disposal germ plasms of diverse origins. It might be asked then if it would not be profitable, even now, to go back ten or even fifteen years and try to bring into our present stocks plasms from other primary isolates, even though the penicillin production from these, at the outset, would be in the range of 100 to 200 U./ml. instead of in the thousands as is expected routinely. May not current developments be limited possibly by the fact that the cultures now being re-

combined all stem from progenitors that arose not only from a single primary isolate, but from single conidia at more than one point in their geneaology?

Concerning some of the findings reported in this monograph, and even more with reference to results that undoubtedly will appear with increasing frequency in the pages of technical journals, a word of caution should be raised regarding the significance and interpretation of product yield in test fermentations seeded with heterozygous diploids of parasexual origin. Experience with heterokaryons points to the necessity of determining, as best one can, the true character and balance of the microbial growth throughout the course of a fermentation. It is not enough to inoculate with spores or mycelia of known heterologous origin and composition and to assume thereafter that the recorded yield of product reflects the biosynthetic capacity of a continuing population of similar genetic constitution. What develops or emerges within the flask during the course of the fermentation is equally important or more so, and careful analyses of the possibly changing population must be followed. Only in this way can meaningful conclusions be drawn concerning the merit of a culture containing any given mixture of nuclei, as in a heterokaryon, or of any recombination of genetic material, as in the nuclei of a parasexually derived heterozygous diploid.

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LYSOGENICITY IN STREPTOMYCETES

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Introduction

A perusal of the literature bearing on the bacteriolytic activities of microorganisms (Welsch, 1947; 1957*b*, *d*; 1958*b*) shows that the role of a bacteriophage has been suggested sometime in the past, but without much proof, to account for the observed lysis of actinomycete cultures. The literature also reveals the fact that continued propagation of an actinophage in streptomycete cultures sometimes has been called lysogenicity, again without proof that the organisms were lysogenic in the present-day sense of the word.

These facts, and my interest in lysogenic induction (Welsch *et al.*, 1953), led me to study actinophages and lysogenic streptomycetes. In addition, I believed that the system actinophage-actinomycete would supply an interesting host-virus model which, although more complex than the usual one-celled bacterium-bacteriophage, yet would be much simpler than the animal-virus or plant-virus ones (Welsch, 1957*c*). Furthermore, it was hoped that actinophage sensitivity or resistance and prophage carrying would be useful markers for genetic studies and welcome additional properties to help answer the difficult question of relationships among actinomycetes (Welsch *et al.*, 1957; Bradley and Anderson, 1958; Welsch, 1959*a*, *b*).

When I started my work on these problems the occurrence of actinophages in nature was known. The chance infection by actinophages of streptomycin-producing cultures of *Streptomyces griseus*, under industrial or laboratory conditions, had been reported, and several of the viruses involved had been studied. However, no evidence for true lysogenicity in actinomycetes had been adduced.

Woodruff *et al.* (1947), however, had reported: "Several resistant cultures have been selected following exposure of *A. griseus* to the actinophage. Many appear to be lysogenic. One culture . . . always had two or three plaques of lysis in agar slant cultures. . . . The actinophage was capable of multiplying to a slight extent on the resistant isolate."

This description is more suggestive of a phage-carrying culture than of a truly lysogenic one, according to the present-day concept of lysogenicity.

To avoid any misunderstanding, it therefore seems justifiable to begin with a short discussion of what is meant by lysogenicity (for details see the reviews of Lwoff, 1953, Boyd, 1956, and Bertani, 1958).

Lysogenicity

Discovered by Lisbonne and Carrère (1922), lysogenicity in the past was studied mostly at the level of the culture, or bacterial population, and generally regarded as a situation analogous to the state of a healthy germ carrier in animals or man.

It was soon recognized, however, that each individual bacterium isolated

from a lysogenic strain produces a bacteriophage-containing culture (Bordet, 1925) and that lysogenicity is maintained after prolonged cultivation under experimental conditions that destroy free phage or abolish its infectivity (Bordet and Ciuca, 1921; Bordet and Renaux, 1928; Burnet and McKee, 1929; Gratia, 1932; Wollman and Wollman, 1936*a*; Northrop, 1951). Further, it was shown that spores from lysogenic strains, after being submitted to a phage-destroying treatment, retain their ability to produce phage-containing cultures (den Dooren de Jong, 1931). Finally, despite occasional reports to the contrary, which were never confirmed, it proved impossible to liberate phage particles by lysis of washed lysogenic bacteria (Burnet and McKee, 1929; Burnet and Lush, 1936; Gratia, 1936*a*; Wollman and Wollman, 1936*b*, 1938, 1939; Gratia and Welsch, 1939; Northrop, 1951).

All these observations were difficult to reconcile with the idea that a truly lysogenic population was made up of phage-resistant individuals, harboring, reproducing, and excreting a virus that would infect their progeny continuously. On the contrary, they led to the idea that phage is present in truly lysogenic cells in a state altogether different from the usual free virus. It is the capacity to produce phage, and not the phage particle itself, that is transmitted hereditarily and endogenously, from one lysogenic cell to its progeny (Bordet and Renaux, 1928; Burnet and McKee, 1929).

The material support for this heritable property is now called probacteriophage or, in brief, prophage. Lwoff and Gutmann (1949*a*, *b*) have shown, by direct microscopic observation and single-cell isolation, that the production of bacteriophages in a lysogenic population results from the lysis of a comparatively small number of bacteria in which prophage is transformed into active phage. Their observations were confirmed by Clarke and Cowles (1952).

If the spontaneous activation of prophage occurs only in a part of the bacterial population, it can be induced experimentally in nearly 100 per cent of the microorganisms by the application of suitable agents such as ultraviolet irradiation (Lwoff *et al.*, 1950), provided that adequate conditions obtain. These have not been discovered as yet for some lysogenic organisms which, until now, remain noninducible.

In brief, each individual cell of a truly lysogenic culture carries a prophage (the potential ability to lyse and liberate free phages) that is transmitted hereditarily to its progeny, endogenously, at cell division. Lysogenicity therefore can be maintained under experimental conditions that are harmful to free phage or can prevent any possibility of exogenous infection, for example, in a culture medium containing a specific phage antiserum or devoid of adsorption cofactors. In fact, lysogenic cells are resistant to exogenous infection by the phage corresponding to the prophage that they carry. Production of phage in a lysogenic culture occurs whenever a metabolic change, the result of spontaneous or experimental induction, has transformed the prophage into an active phage (maturation) whose multiplication into the induced cell is followed by its lysis and liberation of the free virus.

Clarke's observations (1952), however, have demonstrated that nonlysogenic, phage-sensitive mutants can appear in a lysogenic culture. They can be selected by appropriate procedures and, under suitable experimental conditions,

can overgrow the original lysogenic population. The culture then appears to have been "cured" of its lysogenicity.

When acting upon a sensitive cell, bacteriophages from lysogenic cultures will, according to the prevailing conditions, either multiply within and lyse it (lytic response), or undergo the transformation into prophage (reduction) and transform it into a lysogenic cell, the origin of a lysogenic clone (lysogenization). Such phages are called temperate, while those that are unable to lysogenize their host are called virulent or lytic. The distinction, however, is not absolute since the behavior of the phage-bacterium complex varies, not only with the nature of the virus, but also with that of the host and with the experimental conditions.

Pseudolysogenicity in Actinomycetes

Several workers have noticed that after infection of their cultures by an actinophage, streptomycetes frequently are able to propagate the virus for a long time through repeated subcultures. This often has been interpreted as the result of lysogenization, and such phage-carrying cultures have been called lysogenic. The fact that these presumably lysogenic strains show typical plaques when plated on agar, however, is not in very good agreement with the concept of true lysogenicity. Nevertheless, my first experiments were attempts to isolate truly lysogenic strains from actinophage-treated cultures of streptomycetes.

My first observations were made with a phage φ-G 10 and the sensitive indicator *S. griseus* U 1, a streptomycin-producing strain (Welsch, 1954*a*). Both were obtained through the courtesy of J. Ungar, Glaxo Laboratories, Greenford, England. I next used several actinophages isolated directly from natural substrates (Welsch *et al.*, 1955; Welsch, 1956*d*, 1957*e*) and purified by serial isolation from single isolated plaques. A virus designated φ-17 and the corresponding indicator S-17, a representative of *S. chrysomallus*, were studied in particular (Welsch, 1957*a*). Initially, this virus was thought to be highly specific for actinomycin C-producing strains of *S. chrysomallus* (Welsch, 1956*c*), but was later found to have a broader spectrum (Welsch *et al.*, 1957; Welsch and Pinkaers, 1957), including a large number of organisms belonging to the *griseus* group as defined by Corbaz *et al.* (1957). Its properties were described elsewhere (Welsch, 1955, 1956*a*; Welsch and Minon, 1955; Lhoest, 1957).

General techniques. Unless otherwise stated, all cultures were made in peptone-meat extract broth or agar and incubated at 25° C., cultures in liquid medium being shaken. Enumeration of viable bacteria, infectious centers (whole cultures), and free phage particles (culture filtrates or thymol-sterilized supernatants) were made according to the double-layer technique of Gratia (1936*b*), as fully described by Welsch *et al.* (1953). Spore suspensions were obtained by lightly scraping the growth from 5 or 6 day-old cultures on asparagine-peptone agar made in Roux's bottles, in approximately 20 ml. of distilled water. The suspensions were left standing in high narrow tubes for 2 hours, thus allowing the heavier elements to sediment at the bottom and the lighter clumps to accumulate at the air-water interface. The more homogeneous middle part, carefully removed with a pipette, was used exclusively as an indicator for the enumeration of plaques.

Since the results obtained with the different host-virus systems examined are very similar, it will be convenient to summarize them together.

Observations made at the culture level. Broth cultures of the streptomycetes, after addition of the actinophage, either lyse completely within 24 to 36 hours, or assume a more or less pronounced homogeneous turbidity instead of their usual flocculent appearance. Microscopical, rodlike elements and very short, slightly branched filaments are seen instead of the typical growth, the aspect observed being similar to the one described by Koerber *et al.* (1950).

At that moment the phage titer is high: 10^9/ml. or more. However, if serial dilutions of the cultures are plated on agar, colonies do appear. Their numbers, however, show no correlation with the dilution factors. They are much fewer than expected at low dilutions (TABLE 1). This demonstrates that a large number of the microorganisms surviving in the phage-containing broth are, nevertheless, virus-sensitive and lyse on agar, unless the sample plated is so diluted that they can escape infection.

TABLE 1

ABSENCE OF CORRELATION BETWEEN DILUTION FACTOR AND VIABLE COUNT IN CULTURES OF *S. CHRYSOMALLUS* S-17 CARRYING ACTINOPHAGE φ-17

Nature of sample plated	Phage titer (10^8/ml.)	Number of colonies/ml. at dilution						
		10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Infected culture postlytic growth:								
(*a*) 24 hour-old	93	—	67	35	—	7	—	—
(*b*) 12 day-old, stationary	97	652	—	88	—	3	—	—
(*c*) 5 day-old, shaken	89	—	—	513	—	423	72	11
Fourth serial subculture in broth of (*c*), 2 days old	27	85	76	38	150	270	53	9

When phage-infected broth cultures are left on the shaker after complete or partial lysis has occurred, they become the site, within 2 to 4 days, of a heavy postlytic growth, either of the normal flocculent type or of the homogeneously turbid type. If the lysed cultures are left stationary, the postlytic growth occurs within 8 to 12 days as a pellicle bearing aerial mycelium and conidia that are easily broken up and resuspended in the broth.

The phage titer remains unchanged or decreases a little during the period of secondary growth. It is practically impossible to wash the postlytic growth free from phage; moreover, some of its cells are already phage-infected (Welsch, 1956*b*).

When the postlytic growth, after being washed several times, is diluted and plated with conidia of the indicator strain, large and regular plaques are formed, while those obtained under similar conditions with the supernatant or the washings are smaller and of more variable size (FIGURE 1). This is quite understandable, since some variable time must elapse before any free phage particle has had a chance to be adsorbed on a sensitive cell and start the virus reproduction cycle, whereas complexes produce phage almost immediately.

Plated on agar after several washings, the postlytic growth lyses completely

at low dilution, a few isolated colonies appearing only after several days. It gives rise to a confluent growth spotted with more or less numerous plaques at intermediate dilutions (FIGURE 2). Isolated colonies are seen at high dilutions, but again their numbers show no correlation with the dilution factors (TABLE 1). It therefore follows that they are derived primarily from phage-sensitive cells, although the postlytic growth occurred in a fluid containing large amounts of virus.

After serial dilution in broth, the postlytic growth gives rise to cultures of normal appearance in which free phage is produced. The virus can be propagated by subculturing these cultures in broth for at least 16 passages, provided that a heavy inoculum is used (1:10). With smaller inocula, the phage titer decreases at each step until eventually no more virus is found. The same

FIGURE 1. Postlytic growth from a culture of *S. chrysomallus* S-17 infected with actinophage φ-17, washed 8 times and resuspended in saline. *Left side:* washed suspension diluted to 10^{-4} and plated (0.5 ml.) with 0.5 ml. of a conidial suspension from S-17 (10^6/ml.). *Right side:* eighth washing, diluted at 10^{-2} (0.5 ml.) and plated as above. Notice the large and regular plaques from "complexes" and the smaller and more variable ones from free phage.

results are observed when a synthetic medium of glucose, asparagine, phosphates, and calcium chloride is used instead of broth. However, the omission of calcium from this culture medium hastens the disappearance of the phage. The protective action of calcium on several *griseus* actinophages is well established (Koerber *et al.*, 1950; Perlman *et al.*, 1951; Alexander and McCoy, 1956), and the role of divalent cations as adsorption cofactors was demonstrated by Walton (1951).

As long as the subcultures are producing phages, they give rise to a confluent growth spotted with plaques when they are plated on agar at a suitable dilution (FIGURE 3). Here again, viable counts show a lack of correlation with dilution factors (TABLE 1).

These observations show clearly that: (1) individual members of a population of actinomycetes that escaped lysis and their progeny (postlytic growth and subcultures therefrom) are able to withstand a high concentration of virus in

broth, whereas they are lysed on agar; and (2) whenever phage is produced by subcultures of a population exogenously infected, at least some of the cells prove to be virus-sensitive when tested on agar.

If it is assumed that lysogenization has occurred and that phage production is chiefly from lysogenic cells, one must then admit that either a great number of the lysogenic organisms from broth are induced when transferred on agar, or else that they continuously throw off phage-sensitive mutants.

Neither of these interpretations, however, satisfactorily explains why serial subcultivation from small inocula leads to the ultimate disappearance of the phage.

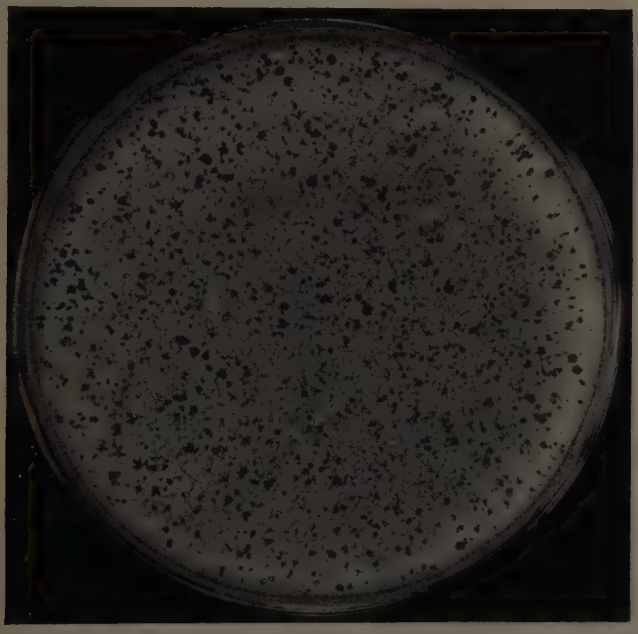

FIGURE 2. Postlytic growth of *S. chrysomallus* S-17 washed 8 times and plated at dilution 10^{-2}.

However, true lysogenicity being a cell property, a study limited to the level of the culture or population can be misleading. The properties of the postlytic growth therefore must be analyzed by examination of single isolated colonies, each presumably being derived from a single microorganism that can, in turn, be submitted to further isolation when showing evidence of heterogeneity.

Observations made at the isolated colony level. Single isolated colonies, obtained under the conditions described under the section *Observations made at the culture level*, show different aspects (FIGURES 4 and 5) and behave in different ways. The same also applies to colonies recovered after plating a heavy suspension of conidia from the indicator strain with a low dilution of actinophage.

For our present purpose, it will suffice to distinguish three types of colonies. Those of type *a* are flat and translucid. They do not grow when transplanted in broth. They vanish completely within a few days, and large amounts of

phage can be recovered from their site on the plate. Colonies of type *b* may be quite normal morphologically or may differ from the parent in size, shape, color, texture, aerial mycelium, and conidia production. In any case, however, they show evidence of being phage-infected, since they undergo a partial lysis,

FIGURE 3. Tenth serial passage in broth from postlytic growth of *S. chrysomallus* S-17 spun down after 2 days and plated at dilution 10^{-3}.

FIGURE 4. Isolated colonies from *S. griseus* U 1: (*a*) from a normal culture; (*b*) from a culture lysed by φ-G 10 (notice small size and absence of aerial mycelium); and (*c*) from the postlytic growth (notice Type *a* and *b* colonies).

sometimes limited to the aerial mycelium, either sector shaped or of a moth-eaten type. Transplanted into broth they grow into normal-appearing cultures in which free phage is produced. Colonies of Type *c* may have any of the different aspects displayed by those of Type *b*, but do not show visible signs of being phage infected.

When they are crushed and streaked on agar or when their conidia are similarly treated, the initial denser parts of the streak rarely lyse completely

and sometimes give a confluent growth with plaques but, most often, a continuous healthy-appearing growth. The more dilute parts of the streak very exceptionally show one or two colonies of Type *a*, not rarely a few of Type *b*, but mostly of Type *c*. When the latter, in turn, are submitted to isolation on agar, they generally produce colonies of Type *c* only which, when inoculated in broth,

FIGURE 5. Isolated colonies from *S. chrysomallus* S-17: (*a*) from a normal culture; (*b*) from a culture lysed by φ-17 (mostly Type *a* colonies); and (*c*) from postlytic growth (crateriform colonies of Type *b* and *c*).

develop into normal-looking cultures containing no phage, whose viable counts show perfect correlation with the dilution factors. Organisms from these broth cultures, plated on agar with conidia of the sensitive indicator, do not form colony-centered plaques. Grown with the indicator in mixed broth culture, they produce no actinophage. In other words, they carry no free phage and are not lysogenic.

The examination of a large number of phage-free isolates derived from the postlytic growth of *S. chrysomallus* S-17 and its subcultures shows that a great

many of them are as sensitive to the virus as is the original strain. Very few are fully resistant, but many are partly resistant.

Koerber *et al.* (1950) have shown that fully resistant mutants are obtained easily for phages of their Group I, but that this is difficult for phages of their Group II. It appears that all phages studied by us belong to their Group II. Later, van Alstyne *et al.* (1955) also reported that only part of the colonies developing from phage-lysed cultures are fully resistant.

Plated with the phage, the partly resistant variants generally form small and turbid plaques in contrast to the large and clear ones seen on the parent strain (FIGURE 6). The efficiency of plating of φ-17 on the partly resistant organisms is also much lower than on S-17 (FIGURES 6 and 7). This might be due to poor adsorption and consequently to low infectivity, but would be

FIGURE 6. Action of φ-17 on *S. chrysomallus* S-17 and variants isolated from its postlytic growth. Plate, *upper left*: S-17, phage at 10^{-7}; other plates: 3 different partly resistant isolates, phage at 10^{-2}.

expected equally if the partly resistant cells were, in fact, susceptible to infection, not by φ-17, but by a rare host-range mutant derived from it.

The occurrence of a stable mutant φ-17/5 has, in fact, been demonstrated in all stock preparations of φ-17 (Welsch, 1954*b*). It is characterized by its broader host range, which includes a number of *S. griseus* strains that produce no streptomycin and are resistant to φ-17 (Welsch *et al.*, 1957).

Some of the partly resistant isolates regenerate φ-17 in serial broth cultures with a high yield, but the efficiency of plating of their culture filtrates always remains much higher on S-17 than on the homologous host. In such cases, there is no selection of host-range mutant and the qualification "partly resistant" applies well to the strain whose rate of infection is low, but whose burst size is high.

With other partly resistant isolates, the regeneration of φ-17 is very poor in broth cultures from which it disappears even after a few serial passages. However, the actinophage can be sampled from a plaque occurring on a layer of the

partly resistant organism, diluted and plated with the same host. Thereafter the procedure can be repeated indefinitely. The phage thus produced has about the same efficiency of plating on its host, on S-17, and on S-5, and therefore resembles the mutant φ-17/5 in its host-range; however, it differs from it in stability. One or two passages on S-17 are enough to have it replaced by a virus showing all the properties of the original φ-17. In the present state of our knowledge it is impossible to decide whether the extended host range of the virus produced by those partly resistant isolates is the result of a mutation or of phenotypic changes.

FIGURE 7. Spot-testing of a stock preparation of φ-17 at various dilutions (10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8}, starting from bottom left and proceeding counterclockwise) upon *S. chrysomallus* S-17 (*upper left*) and 3 phage-free isolates from its postlytic growth.

Discussion. Our observations show that after actinophage lysis of an actinomycete culture the surviving population is quite heterogeneous, consisting of organisms from which will evolve clones endowed with variable degrees of virus sensitivity, including some fully sensitive and resistant, but among which no truly lysogenic ones are found.

The serial mass transfer of such a heterogeneous population in broth and, to a lesser extent on agar, however, may result in the continued propagation of an actinophage through many subcultures, a situation that may be called pseudolysogenicity.

The occurrence of partly resistant organisms, producing much phage but being infected and lysed at a low rate only, explains how the virus-host association can be maintained for a long time. In addition, several other factors may help to prevent a quick elimination of the phage-sensitive elements from the heterogeneous population.

Sensitive strains are not lysed by actinophages at some stages of their growth cycle, such as aerial and submerged spores (Rice, 1953) or aged mycelium

(Woodruff *et al.*, 1947; Newbould and Garrard, 1954). Environmental conditions also may reduce lysis: for example, the degree of oxygenation (Carvajal, 1953). The virus also can be bound temporarily to resistant organisms (van Alstyne *et al.*, 1955) and meanwhile prevented from attacking sensitive cells. Finally, heterokaryosis may be an important factor in maintaining the heterogeneity of the population during serial cultivation from large inocula.

True Lysogenicity in Actinomycetes

Since actinophages directly isolated in the free state from natural substrates were unable to lysogenize their hosts, screening tests were devised to discover naturally occurring lysogenic actinomycetes. Several such strains were recognized, as reported in a short note (Welsch, 1956*e*) and in a more detailed report whose publication was unfortunately delayed (Welsch, 1958*a*).

At about the same time, Alexander and McCoy (1956) mentioned that their actinophage W-1A had been isolated from a lysogenic culture, *S. griseus* 1945, but no information was given as to the behavior of this organism. Another possibly lysogenic streptomycete was described by Shirling (1956) but, unfortunately, its study was hampered by the lack of a suitable indicator strain on which plaques could be formed and enumerated. In fact, the author wrote, "From the data now available it cannot be stated with certainty whether S-77 and its variants are lysogenic in the sense that all of the mycelium carries prophage internally."

Later, lysogenic streptomycetes were reported by Bradley (1957), Rautenstein (1957), and Khavina and Rautenstein (1958).

Screening of naturally phage-carrying strains. The organisms examined come from different sources. A first series comprises nonidentified strains isolated from different natural substrates and at different times between 1934 and 1947 in the course of our work on the antibiotic and bacteriolytic activities of actinomycetes (Welsch, 1947; Ghuysen, 1957). After isolation, these organisms were continuously maintained on agar slants at 2° to 4° C., with transplantation every 3 or 4 months alternately on different culture media: Czapek-asparagine agar, mushroom extract-lactose agar, asparagine-peptone agar, keratose-casein agar, peptone-meat extract agar.

A second series comprises chiefly actinomycin-producing organisms from the *Institut für Spezielle Botanik*, Zürich, Switzerland (Welsch *et al.*, 1957).

A third and last series consists of freshly isolated strains, especially from samples of soils recently collected from central Africa. Its study is still in progress.

Strains are tested in groups of 24. Broth cultures of each are incubated at 25° C. for 48 hours and centrifuged, and the supernatants are decanted. A small crystal of thymol is added and the sample is stored in the cold for at least 48 hours before being tested.

Each group of 24 supernatants is spot-tested on each of the corresponding 24 strains and, sometimes, on a few others also. A multiple-point inoculator (FIGURE 8), slightly modified from the description of Beech *et al.* (1955), is used to deposit at once in a regular pattern 24 droplets, one from each supernatant, on each one of the series of 24 or more agar plates each inoculated with one of the indicators.

After an incubation of 24 to 48 hours, the indicators have grown into a continuous layer on which clear zones may appear surrounding the point of impact of one or another of the inoculating rods (FIGURE 9).

The observation of such a clear zone does not mean necessarily that an actinophage was present in the droplet tested: an antibiotic, for instance, would give the same appearance. However, even at this stage the presence of a virus can be suspected strongly when the clear zone has a lobulated margin or manifestly results from the coalescence of several discrete plaques.

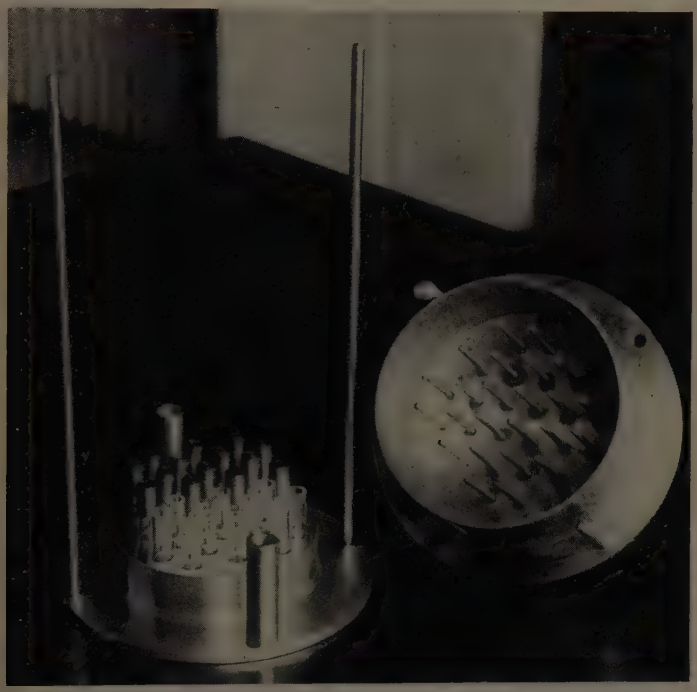

FIGURE 8. Multiple-point inoculator used for spot-testing phage-containing fluids (adapted from Beech *et al.*, 1955).

In any case, the supernatants giving a positive result in the preliminary screening test are plated on agar at several dilutions with conidia from the apparently sensitive organism or organisms.

If normal growth of the indicators occurs in the presence of whatever dilutions of the supernatant used, the test for phage carrying is considered to be negative.

On the contrary, formation of typical plaques unequivocally indicates the presence of an actinophage, and the producing culture is retained for further testing.

Sometimes no plaques are seen, but the growth of the indicator is inhibited by low dilutions of the supernatant. Although such a result suggests the action of an antibiotic, our experience has shown that it may be due to a phage. Therefore, in such a case the active supernatant is introduced (0.5 ml.) in a broth culture (10 ml.) of the sensitive strain, which is centrifuged after an

incubation of 48 hours. Its supernatant then is tested for plaque production. If no plaques are formed, the same procedure is repeated once or twice in succession before it is concluded that no virus is present.

Tests of true lysogenicity. Two general tests were used to recognize, among the phage-carrying organisms detected, the truly lysogenic and the pseudolysogenic ones.

(1) On the basis of the experiments described above under the section *Pseudolysogenicity in Actinomycetes*, I thought that true lysogenicity could be assumed safely when the production of an actinophage by a given organism was maintained after ten isolations on agar in series, each time from a single well-isolated colony. I realize of course, that the conclusions would have more

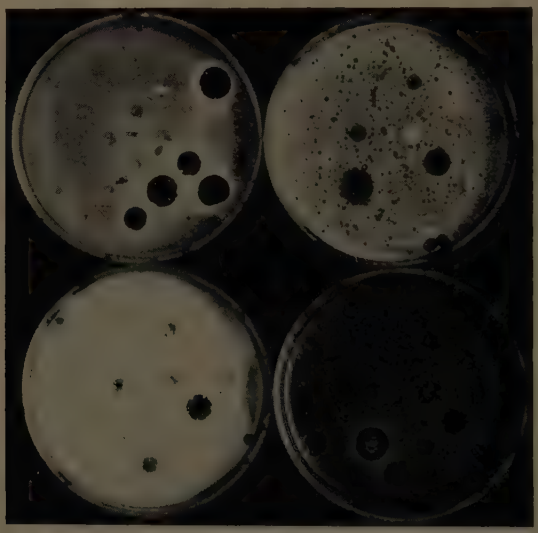

FIGURE 9. Results of the spot-testing of the supernatants from 24 actinomycete cultures on 4 different indicators: S-80 (*upper left*), S-84 (*upper right*), S-100 (*lower left*), and S-88 (*lower right*).

weight if single-cell isolation was performed under the microscope with a micromanipulator. However, it may be recalled that in previous experiments pseudolysogenic organisms always were freed from the phage they carried after only two, or at most three isolations in series.

(2) The presumably lysogenic organisms must show no plaques when plated with the phage derived from them and regenerated on a suitable indicator, even at low dilution. It is very important as will be shown later, to perform this test with the lightest possible spore suspension that will give a continuous layer of growth.

Additional and more stringent tests of true lysogenicity also have been applied, but to-date only to a few of the strains. They are: maintenance of phage production after repeated subcultures, each from a small inoculum, (*a*) in a synthetic medium without added divalent cations, (*b*) in the presence of citrate, or (*c*) in the presence of a specific phage-inactivating antiserum.

Tests *a* and *b* confirmed the occurrence of true lysogenicity in the 4 strains to which they were applied. Test *c*, which has been applied thus far only to a representative of *S. griseus* S-55 L, showed that the capacity to produce phage was retained by the microorganism after 6 cultures made in series in the presence of an efficiently phage-inactivating serum (Welsch, 1958*a*).

Occurrence of truly lysogenic actinomycetes. It must be emphasized that the results of the screening are dependent on the chance occurrence of suitable indicators among the cultures included in the test. For this reason, numerical data on the frequency of truly lysogenic organisms found have no absolute significance.

Among 96 cultures of our collection, tested in groups of 24, about one half gave a positive preliminary screening test (clear zone when spot-tested). One fifth of these (about 10 per cent) were actually phage-carrying, the fact being established by immediate formation of plaques in two thirds of the cases, but only after one or more passages in broth cultures of the indicator in the remaining third. All of the phage-carrying cultures thus discovered were truly lysogenic according to criteria already described.

A series of 47 selected organisms was studied next and, in this case, complete cross-testing of all cultures was performed. This collection, gathered to determine whether sensitivity to φ-17 was linked with actinomycin production or with affinity for the *griseus* group (Welsch *et al.*, 1957), comprised collection strains and freshly isolated strains from various sources: 34 came to us from the Zürich collection, 11 were isolated by us, and two were respectively obtained through the courtesy of G. Giolitti, Montecatini, Milan, Italy, and P. de Somer, University of Louvain, Louvain, Belgium.

Among these organisms four were representatives of *S. griseus*, producing no streptomycin and sensitive to φ-17. The others were actinomycin-producing streptomycetes that were thus classified by Corbaz *et al.* (1957): Group I, chromogenic, spores *cinereus*, actinomycin X produced, 6 strains, including the organism provided by G. Giolitti and 2 collection representatives of *S. antibioticus*, namely ATCC 8663 (original) and (NRRL) ATCC 10382; Group II, chromogenic, spores *griseus*, actinomycin X produced, 1 strain; Group III, nonchromogenic, spores *cinereus*, actinomycin I produced, 9 strains, including the collection strain *S. parvullus* ATCC 12430 (original); and Group IV, nonchromogenic, spores *griseus*, one strain producing actinomycin X (a collection strain: *S. parvus*, NRRL B-1455 = ATCC 12429) and 26 strains producing actinomycin C, including a collection strain: *S. chrysomallus* ATCC 11523 (original), the organism provided by P. de Somer and 7 isolates from our collection.

With this series of strains, chiefly comprising antibiotic-producing organisms, the spot test gave slightly more than 50 per cent positive results. However, in a single instance positive results were due to an actinophage. The carrying strain, a representative of *S. chrysomallus* numbered z-28 in the series, was found to be truly lysogenic by the criteria described previously. It was the only strain of Group IV to be fully resistant to actinophages φ-17 and φ-17/5, a property that is, very likely, ascribable to the prophage which it carries. As a matter of fact, the phage it produces, φ-z 28 L, appears to be related to φ-17,

but its host range is narrower, and it is not inactivated by anti-φ-17 immune serum.

Tests performed on freshly isolated streptomycetes are still in progress. From the data now available, it seems that the frequency of lysogenic isolates is lower than in the first series of strains examined.

Properties of lysogenic strains. The lysogenic organisms tested produce only a small amount of free phage in broth cultures inoculated from a single isolated colony, titers of 10^5 to 10^6 plaques/ml. at most being observed after 48 hours. In cultures heavily inoculated with conidia and vigorously shaken, titers up to 10^8/ml. can be obtained. When cultures of a given strain are made under rigorously standardized conditions, it is found that the quantity of virus produced is constant, a fact that is in good agreement with true lysogenicity. However, owing to the peculiar mode of growth of streptomycetes, giving rise, even in shake-flask cultures, to microcolonies of variable size, it was difficult to establish a constant numerical relation between viable counts and free phage.

Attempts were made, but thus far on a small scale only, to lysogenize several indicators with actinophages from lysogenic strains. It was hoped that these viruses, in contradistinction to those directly isolated as free particles from natural substrates, would behave as temperate phages. However, they were able, as were the former, to associate with the bacteria as pseudolysogenic cultures. Among the colonies isolated from their postlytic growth, a few appeared to have been truly lysogenized, but the data available at present are not conclusive.

Actinophages from lysogenic strains are generally able to act on several indicator hosts, and their individual efficiency of plating varies widely. When a given virus has been passed, once or in a series, upon a given host, it generally shows the same host range as the original, but its efficiency of plating for the various indicators is completely changed, being always highest for the host. Possibly, this property might be helpful in ascertaining the degree of relationship between related strains of streptomycetes.

The variations of efficiency of plating occur and revert quickly after even a single passage on the appropriate host. The heterogeneity of the phage population that they reflect is therefore more likely to be due to phenotypic changes than to mutations.

As an example of this behavior, TABLE 2 shows the efficiency of plating, upon several indicators, of φ-55 L-suspensions obtained after regeneration on different hosts.

Attempts were made to define the conditions under which lysogenic streptomycetes could be induced. To this aim, suspensions of newly germinated conidia in synthetic medium were submitted in the dark to ultraviolet irradiations of different intensities and for different lengths of time. The irradiated organisms were introduced in broth and incubated in darkness on the shaker at 25° C. No evidence of lysis or of an abnormally high production of free phage was ever observed.

Heavy suspensions of conidia were spread on agar and the plates submitted to ultraviolet irradiations as above. The plates then were incubated in the dark. Most frequently a continuous layer of growth was observed without any evidence of lysis.

In some cases, however, the actinomycete layer was spotted with a number of small, almost pinpoint holes that increased slightly upon further incubation (FIGURE 10). When this phenomenon occurred, it was observed on all the

TABLE 2

VARIATIONS IN THE EFFICIENCY OF PLATING OF φ-55 L WHEN PRODUCED BY AND TESTED ON DIFFERENT HOSTS

Highest dilution at which plaques are still formed on the indicators shown in the table	Suspensions of actinophage φ-55 L produced by the following hosts				
	S-9; S-14	S-10; S-64	S-76	G	S-55 L
10^{-8}	—	S-10; S-64	S-76	G	S-10; S-64
10^{-7}	—	—	—	S-10; S-64	S-9; S-14
10^{-6}	S-9; S-14	S-9; S-14	—	S-9; S-14	—
10^{-5}	—	—	S-10; S-64	—	G
10^{-4}	S-10; S-64	G	S-9; S-14	—	S-76
10^{-3}	—	S-76	G	S-76	—
10^{-2}	G	—	—	—	—
10^{-1}	S-76	—	—	—	—

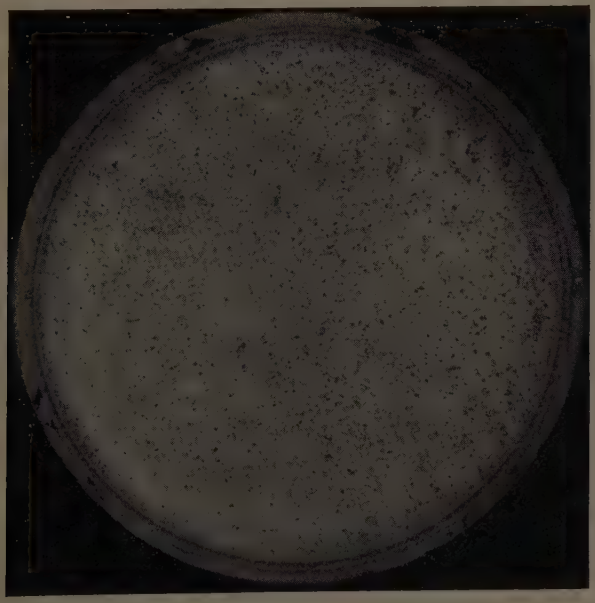

FIGURE 10. Very small plaques appearing on the continuous growth of *S. chrysomallus* S-17 when it is plated as a concentrated suspension of conidia (spontaneous induction?).

plates of a given lot, whatever the dose of ultraviolet light received, including the nonirradiated controls. In fact, this happened whenever the plates had been seeded very heavily. I interpret these plaques as the result of spontaneous induction. Such a phenomenon could not occur with ordinary bacteria since, in order for a plaque to appear, the lysogenic bacterium must be surrounded by sensitive cells. However, in the case of streptomycetes the size of a micro-

colony can be such that its lysis through spontaneous induction would make a tiny, but visible hole on the confluent growth layer.

Several of the lysogenic actinomycetes are sensitive to virulent actinophages. I have not yet studied the possibility of their induction by virus infection, a phenomenon observed by Bradley (1957). However, occasional observations have shown an abnormally high production of the temperate phages in lysogenic cultures treated with a virulent phage to which they were sensitive.

Discussion

A comparison of my findings with those of other workers is difficult, since their publications are either short notes (Bradley, 1957) or papers in Russian with English summary (Rautenstein, 1957; Khavina and Rautenstein, 1958).

There is complete agreement, however, on the fact that true lysogenicity is not rare among actinomycetes, this condition being found in collection strains as well as in organisms freshly isolated from natural substrates.

All the actinophages that I have isolated and studied so far, whether they were obtained directly from natural substrates or derived from lysogenic organisms, have shown a more or less extensive host range, but chiefly within the *S. griseus* group as defined by Corbaz *et al.* (1957). Some of them, however, show a limited activity on species outside the group when used at a high concentration. In fact, repeated attempts, including enrichment cultures, have been made and are still in progress to obtain actinophages acting upon several other well-defined species of actinomycetes, but thus far without success.

In addition to lysogenic *S. griseus*, Bradley (1957) obtained one lysogenic *S. olivaceus* and several unidentified lysogenic isolates, but could not demonstrate lysogenicity in the representatives of *S. coelicolor* and *S. cyaneus* tested. Rautenstein (1957) and Khavina and Rautenstein (1958) state that 53 per cent of the *Act. olivaceus* and 43 per cent of the *Act. levoris* tested were lysogenic. In addition, they obtained one lysogenic strain of each of the following species: *Act. diastaticus*, *Act. cacaoi*, *Act. candidus*, *Act. griseus*, *Act. antibioticus*, *Act. scabies*, and *Act. odorifer*.

Apparently Bradley (1957) obtained negative results, as I did, when trying to induce lysogenic cultures by procedures that are effective with other bacteria. However, he succeeded in obtaining their induction by virulent phages. Some of my observations possibly involve a similar mechanism, but need further research.

Rautenstein (1957) writes: "The indicator culture . . . often influences substantially the lytic properties of the given phage." This probably refers to changes in the efficiency of plating similar to those which I observed. Rautenstein adds, "This influence becomes especially stressed when the indicator is itself lysogenic." This possibly refers to the induction of one lysogenic organism by a virus produced by another lysogenic strain.

There is a last point, however, about which there is some lack of agreement. Bradley (1957), who used lysogenic strains purified by several isolations made in series, and the Soviet workers, whose techniques and criteria for true lysogenicity are not precisely known to us, state in Bradley's words, "Some of the temperate phages are able to attack the lysogenic strain from which they were

derived." Personally, I found that conidia of the lysogenic organisms, plated at the highest possible dilution giving a continuous layer of growth, with high-titered preparations of the actinophage that they produce, do not show plaques. On the contrary, plaques do appear when they are plated as a concentrated conidial suspension. However, this, occurring in the absence as well as in the presence of added phage, is interpreted as the result of spontaneous induction rather than of exogenous infection.

Conclusions

True lysogenicity occurs not infrequently in actinomycetes. It must not be confused, however, with pseudolysogenicity, which is very often observed in actinomycete cultures that have been infected exogenously with an actinophage, either virulent or derived from a truly lysogenic organism.

Pseudolysogenic cultures submitted to isolation give normal and abnormal colonies, among which some are obviously phage-infected and phage-sensitive since they undergo complete or partial lysis. The others, showing no sign of phage-infection either are, in fact, phage-free or yield phage-free clones upon further isolation.

Many of these clones are fully sensitive and a few fully resistant. A great number of them are partly resistant, their cells being phage-infected at a low rate only, but reproducing the virus in high yield. The occurrence of these partly resistant organisms, together with a number of accessory factors, explains the fact that production of the phage can be maintained through a great number of subcultures made in series.

Pseudolysogenic cultures are easily "cured" by repeated subcultures from small inoculates, and much more quickly if a medium either devoid of calcium or enriched with a specific phage-inactivating antiserum is used.

In contradistinction, truly lysogenic cultures submitted to isolation give only healthy colonies showing no sign of phage infection, but producing free phage when grown in broth.

The capacity of such cultures to produce the virus is retained after an indefinite number of isolations and after repeated subcultures in the absence of calcium or in the presence of phage antiserum.

The lysogenic organisms are resistant to the actinophage that they produce after induction although, according to Bradley (1957) and Rautenstein (1957), they may show a low degree of sensitivity.

Each phage from a lysogenic strain has its characteristic host range which it retains qualitatively unchanged after it has been passed through different hosts. However, different preparations of a same actinophage, each produced by a different host, show different efficiencies in plating on the different indicators.

Under the various experimental conditions used, none of the lysogenic strains tested was induced by ultraviolet irradiation. Several observations suggest that induction might be achieved through the action of a virulent phage, a procedure that was, in fact, successfully used by Bradley (1957).

Tiny plaques may appear on agar plates heavily seeded with a lysogenic strain. They are regarded as resulting from the spontaneous induction and lysis of microcolonies.

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Discussion of the Paper

W. Gold (*Bzura, Inc., Keyport, N. J.*): I have been struck by certain parallels between the exchange of nuclei in heterokaryosis and the infection of susceptible cells by actinophage.

For heterokaryosis to occur, a hypha must contact a compatible hypha, fuse with it, and form an opening through which a nucleus passes. The nucleus multiplies in the foreign cytoplasm, its offspring then segregate into individual conidia, and finally resume their original identity.

To become infected by actinophage, a hypha must make contact with a compatible phage that probably forms an opening in the hyphal wall through which its nucleus passes. The phage nucleus, or prophage, multiplies in the foreign cytoplasm for awhile, after which its descendants segregate and again form complete, infectious phage units.

The analogy suggests at least one line of investigation that might be fruitful. It has been seen that *p*H and medium composition control the rate of cell infection by phage. It is possible, and even probable, that they play an equally important role in determining the compatibility of strains that can enter into heterokaryosis. Experimental conditions, then, should be controllable, either to permit or to prohibit nuclear exchange, as desired. Investigation might determine also the biophysical and biochemical changes in the cell surface that cause increases or decreases in compatibility.

Could not this comparison be extended to include lysogenicity and, possibly, to explain it in terms of some concepts arising from the study of heterokaryosis? Might not studies of heterokaryosis, diploidy, and dominance be given new direction by some of the work already done on lysogenicity?

EFFECTS OF THE MEDIUM AND ITS COMPOSITION ON THE ACTIVITIES OF ACTINOPHAGE FOR *STREPTOMYCES GRISEUS**

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In several papers[1-5] Welsch has reported the difficulties he encountered in attempts to produce lysogenic strains of *Streptomyces griseus* and of some actinomycin-producing streptomycetes. These difficulties include: (1) poor adsorption of phage to cells; (2) recovery largely of sensitive isolates from secondary growth in lysed cultures; (3) carrying of the phage by some partly resistant colony isolates through several transfers; (4) failure of small phage inocula to lyse cultures of cells completely; (5) decrease in phage yield with increase in age of cell inoculum; (6) failure to account for as much as 50 to 80 per cent of the phage added in some experiments; and (7) irregularity in size and shape of plaques of certain phage strains. The difficulties led to the isolation of many false positives and made their elimination tedious. Several mutants of the actinomycin producer that have been isolated seem to have an increased resistance to phage. These strains give markedly reduced numbers of plaques when used in plates and generally produce diminished crops of phage in liquid culture. In some instances, it may be true that the streptomycetes are inherently somewhat resistant even to the phages that parasitize them. However, the oddities thus far described also may result from other experimental conditions unfavorable to the phages and not directly related to the cells.

My discussion will present evidence, hitherto unpublished, showing that the above-mentioned difficulties with a phage of *S. griseus* can be minimized and sometimes eradicated if attention is paid to such variables as *p*H, salt composition, and salt concentration of the liquid and solid media. I shall attempt to generalize from these results and from the published results for other phage-cell systems to the phages of the actinomycin-producing streptomycetes.

Experimental Methods and Materials

All phages used were identical in their activity on indicator strains of *S. griseus* and had been purified by picking single plaques through 3 serial transfers. The culture usually used, number S3464 in the Rutgers collection, was asporogenous on the media used and grew as filaments, rather than as pellets, in liquid culture. It was maintained by a 1-ml. transfer every 24 hours to 100-ml. fresh medium in shaken flasks at 22° C.

The medium originally contained 1 per cent cerelose, 0.5 per cent beef extract, and 0.5 per cent yeast extract, with or without 1.5 per cent agar. Later, the cerelose was reduced to 0.5 per cent, and peptone was substituted for the yeast extract.

* The work reported in this paper was done in the Department of Microbiology at Rutgers University, New Brunswick, N. J., during tenure of a Waksman-Merck Postdoctoral Research Fellowship.

The phages were counted by mixing the samples with 0.5 ml. of the 24-hour-old shake culture of cells and 5 to 10 ml. of the agar mentioned above. In later experiments, sufficient calcium chloride solution was added to the plate to make its concentration M/100 after addition of the agar. (When the calcium chloride was added to the agar before sterilization, it precipitated as the phosphate during autoclaving.) In some experiments, the samples were plated in a top layer poured over a base layer of agar containing calcium chloride. The plates were incubated at 28° C.

Effect of pH on Phage Activity in Plates and in Liquid Media

On unbuffered agar, initially at *p*H 5.9 to 7.2, the reaction rose to above *p*H 8 in 4 days, and phage plaques took 2 or 3 days to develop fully. Phosphate buffer, at concentrations up to 0.5 per cent $NaH_2PO_4 \cdot H_2O$, permitted lysis of strain S3464 by undiluted phage lysates streaked over seeded agar plates, but 0.75 per cent or more of buffer inhibited lysis.

Agar media were prepared containing 0.1 per cent phosphate with *p*H values of 5.2 to 7.4 after autoclaving.

Duplicate plates at each *p*H level were inoculated with about 1250 phage particles and 0.2 ml. of a suspension of spores, and were incubated several days at 28° C.

In 24 hours many large, definite plaques were visible on all plates at *p*H 6.5 and 6.85 while, at 48 hours, lysis was confluent and had cleared the plates almost completely. The plates at *p*H 7.2 had smaller plaques at 24 and at 48 hours, with much less confluent lysis. The plates at *p*H 6.0 and *p*H 7.4 had even smaller plaques on both days. At *p*H 5.2 and 5.3 all plaques were very small, and growth was slow and scanty. Afterward, the initial *p*H of all media was kept between 6.5 and 6.8.

In unbuffered liquid culture, sterilized by filtration, the effect of *p*H on phage activity was different, probably because experiments lasted only a few hours instead of days, and *p*H changes were consequently small. Samples of culture S3464 were washed and resuspended in 10 volumes of fresh broth at different *p*H values, then infected with diluted phage No. 3. The mixture was incubated at 28° C., and aliquots were plated at intervals.

Since the burst occurred in all cases at from 3 to 4 hours, the results (TABLE 1) give only the initial and 4-hour counts. It may be seen that the phage becomes incapable of normal multiplication when the *p*H is lower than some value between 5.5 and 5.75. I have had similar experiences[6] with a phage active against *Clostridium madisonii*, and Frisbee and MacNeal[7] report that the *p*H of the medium influences some phages active against *Escherichia coli*. In the case of *C. madisonii* phage, the *p*H controlled only the first few moments of infection, probably the act of penetration of the cell by the phage. The fact that the *p*H of the external medium should have any effect at all on *S. griseus* phage suggests an interference with the phage prior to entry into the cell.

At the higher *p*H levels, there is no interference with phage activity up to *p*H 7.6. This is at odds with the observation in agar plates that phage activity becomes restricted at *p*H 7.2 and more so at *p*H 7.4. However, results of other experiments will explain the discrepancy.

Calcium Requirement for Phage Activity

The experiments above had indicated that concentrations up to 0.5 per cent of sodium phosphate in agar did not interfere with lysis of *S. griseus* by large phage inocula. As a result, 0.1 per cent $NaH_2PO_4 \cdot H_2O$ was added to the cerelose-yeast extract–beef extract agar to buffer it. In a few experiments, run with phage of known count and using this medium, a great number of plates had no plaques at all, and the rest seldom had as many as 10 plaques. The plaques were usually small, irregular, and ill-defined, and frequently doubtful. The phosphate, therefore, does interfere with phage even at levels

TABLE 1

INITIAL AND FOUR-HOUR TOTAL PHAGE COUNTS OF SAMPLES OF STRAIN S3464 INCUBATED WITH PHAGE NO. 3 IN BROTHS AT DIFFERENT *p*H VALUES FOR 240 MINUTES

Experiment number	*p*H of broth	Total infectious centers per ml.	
		Initial	Four hours
21	5.05	—	39
	6.8	22	226
	6.9	18	100
22	5.45	22	7
	6.9	38	>600
	7.65	65	>600
28	5.5	26	9
	5.75	34	ca. 600
	6.4	35	>600

TABLE 2

REVERSAL OF PHOSPHATE* INHIBITION OF PLAQUE FORMATION BY CALCIUM CHLORIDE

	Final molarity of added calcium chloride × 10^4					
	0	2	4	6	8	10
Plaque counts	31†	27	90	111	91	54

* Phosphate concentration is 7.25×10^{-4}.

† Average of three counts; other figures are for single plates.

below 0.5 per cent, but massive inocula of phage can still produce confluent lysis.

An experiment demonstrated that the phosphate acted by removal of calcium and that its suppression could be reversed by addition of calcium.

Diluted phage No. 15 and cells of S3464 were mixed in a large volume of broth with 0.1 per cent phosphate. Samples of the mixture were added to plates containing various volumes of M/100 calcium chloride solution and were diluted to 10 ml. final volume with agar. The plates were incubated at 28° C. TABLE 2 shows that plates with no added calcium had low counts. The highest counts were obtained when calcium and phosphate were of approximately equal molarity. Other monovalent and divalent metal ions were unable to replace calcium.

This explains why the agar plates with a *p*H above 6.8 had smaller plaques.

Phosphate in the agar precipitated calcium more easily at the higher *p*H values. The broth, on the other hand, had no added phosphate, and therefore may have allowed phage activity to proceed unhindered in more basic media.

Citrate inhibited phage at concentrations of M/100 and higher. Dilute phage No. 15 was added to washed cells of strain S3464 resuspended in 10 volumes of plain broth or of broth M/50 to sodium citrate. Aliquots of the mixtures were incubated at 28° C. with additions of M/1 calcium chloride. Initial counts were made at 35 min. and final counts at 270 min. on plain agar plates. In addition, control samples with no added calcium chloride were plated over a base agar layer with 1 per cent calcium chloride.

Table 3 shows that calcium chloride at concentrations of 0.165 M (0.2 ml. added) or greater is completely inhibitory to phage multiplication in plain broth. In the citrate broth, where lower concentrations of calcium chloride

Table 3

Effect of Calcium on Multiplication of Phage in Broth with and without 0.02 Molar Sodium Citrate

ml. of M/1 $CaCl_2$ added per 1 ml. sample	Number of infectious centers per plate			
	Plain broth		Broth with citrate	
	35-min. plating	270-min. plating	35-min. plating	270-min. plating
0	15, 18	—	—	—
0*	40	>300	56	26
0.02	—	—	26	300
0.05	—	—	33	>300
0.07	—	—	52	150
0.1	—	—	70	110
0.2	70	61	61	110
0.4	69	39	70	62
0.6	58	44	61	58
0.8	58	42	66	62
1.0	62	—	71	60

* Samples were plated over a layer of 0.02 per cent $CaCl_2$ in agar.

were used, inhibition is evident even at 0.065 M (0.07 ml. added) salt concentration. The optimum level of calcium chloride to add to 0.02 M citrate is 0.02 to 0.05 M, roughly an equivalent molar concentration.

Interestingly, the initial count of the control with the base layer of calcium chloride agar is about 3 times the initial count of the plates without the base layer. All other initial counts of samples with sufficient added calcium chloride are at least 4 times as high. This indicates that the plain agar does not have sufficient calcium to give a true count of the phage and that calcium chloride, added to the agar at concentrations of at least 0.01 M, will increase the "plating efficiency" of the phage count 3 to 4 times.

The suppression of phage multiplication in broth by calcium at concentrations of M/100 or greater is caused by its interference with adsorption of phage to the cells. In an adsorption experiment, 1 ml. of culture S3464 and 1 ml. of a dilution of phage No. 15 were mixed in the following media: plain broth; broth with 10^{-4} M calcium chloride; broth with 10^{-3} M calcium chloride; and

broth with 10^{-2} M calcium chloride. A control phage was included and diluted in plain broth without cells. After 15 min. of incubation, the mixtures were centrifuged and the fluids plated in duplicate. TABLE 4 shows unmistakably that calcium chloride interferes with adsorption of phage, although not completely, at a concentration of 10^{-2} M. This agrees with the results of van Alstyne *et al.*,[8] who found that 5×10^{-3} M calcium chloride allowed the greatest adsorption. They found less adsorption in their medium, not only as the calcium chloride concentration became greater, but also as the concentration decreased. In my experiment described above, the medium probably contained sufficient salts to prevent the decreased adsorption with smaller amounts of calcium. In experiments to be reported below, performed in very dilute buffer, adsorption decreased as the salt concentrations became too low.

Calcium is required early in the infection cycle. Samples of a mixture of phage and cells were diluted at timed intervals in 1 per cent phosphate buffer at *p*H 6.5 to block the step requiring calcium. They were incubated for 280 min. at 28° C. to allow all infected cells to burst, then total phage counts were made in plates over a base layer of agar with 0.02 per cent calcium chloride.

TABLE 4
EFFECT OF CALCIUM ON ADSORPTION OF PHAGE TO CELLS IN BROTH

Sample	Phage per ml. of supernatant medium
Phage alone	215
Phage with cells	14.5
Phage with cells and 10^{-4} M $CaCl_2$	20
Phage with cells and 10^{-3} M $CaCl_2$	24.5
Phage with cells and 10^{-2} M $CaCl_2$	134.5

TABLE 5 shows that the burst occurred between 2 and 3 hours, so that a period of 280 min. was enough time for all infected cells to burst and to release phage. Interference with burst size by phosphate was evident from the first minute of infection, decreasing as addition of phosphate was delayed.

Plates with added calcium were observed to develop heavy precipitates, presumably of calcium phosphate, as growth of the streptomycete caused the *p*H to rise. This observation and the experiments above explain the decrease in "plating efficiency" as the cell inoculum increases. Cell inocula of 0.1 ml. in a 10-ml. agar plate with certain phage dilutions gave plaque counts of 100 to 200/plate. Increasing the cell inoculum to 0.5 ml., 1 ml., or 2 ml. reduced counts to 5 or 10 plaques/plate with the same phage dilutions. However, when calcium chloride was present at concentrations of M/1000 to M/100 in the agar, not only did the reduction of the plaque counts disappear, but even the highest counts were increased. Plaque sizes increased from 1 mm. or less in diameter to 5 mm. or greater in diameter. Manganese chloride, magnesium sulfate, and zinc sulfate could not replace calcium.

These results suggest a reason for the similar effect obtained with inocula of increasing age over the range of 0 to 24 hours. Since it takes several hours for spores to germinate, and several more hours for them to grow to branched

pellets,[3] the increasing ages represent increasing rates of metabolism, particularly of the protein-derived ingredients of the media. The older inocula can raise the *p*H of the media by release of ammonia much sooner than the younger inocula, precipitating available calcium and stopping phage action sooner.

TABLE 5
DETERMINATION OF THE TIME OF CALCIUM REQUIREMENT BY TIMED BLOCKING WITH PHOSPHATE

Time of sample dilution (min.)	Number of infectious centers per plate	
	In phosphate broth after 280 min.	In plain broth, plated when sampled
1	53.5	—
3	93	—
5	73	—
7	119	—
9	100.5	—
11	221	—
13	244	—
15	274	—
17	>275	—
19	>275	—
21	>275	—
23	>275	—
25	>275	—
40	—	61
120	>275	219
180	>275	>500
240	—	>500

TABLE 6
EFFECTS OF DIFFERENT CONCENTRATIONS OF VARIOUS CATIONS ON ADSORPTION OF PHAGE TO CELLS

Salt	Plaques per ml. of supernatant sample			
	M/10	M/100	M/1000	M/10,000
Buffer	—	—	—	190
NaCl	345	18	81	—
KNO_3	299	22	147	—
$(NH_4)_2SO_4$	216	104	103	—
Buffer	—	—	—	142
$CaCl_2$	—	92	27	79
$BaCl_2$	—	55	68	121
$MgSO_4$	—	71	23	117
$MnCl_2$	—	77	31	129
$ZnSO_4$	—	8	27	130

This would decrease final phage crops, but there would probably be little or no effect on the size of the first few bursts before the *p*H rises.

Effect of Other Salts

While this work was being done, the paper of Puck *et al.*[9] reported the effects of many salts on the adsorption of phages to *E. coli*. Puck and his associates found that all divalent metal ions promoted adsorption of phage to

cells at M/1000 concentration, but not at M/100 or M/10,000 concentrations. The monovalent cations caused greatest adsorption at M/100 concentration, but not at M/10 or M/1000 levels.

The same general results (TABLE 6) were found with phage No. 15 and culture S3464. The salts were diluted in a M/10,000 phosphate buffer of *p*H 6.8. At this concentration no visible precipitate formed with the divalent salts. The buffer itself did not promote adsorption.

Thirty minutes after mixing the phage and cells with the salts solution, the mixtures were centrifuged, and the supernatant fluids were plated in duplicate.

The effect of sodium chloride has been reported also by van Alstyne *et al.*,[8] who added enough salt to wash surface phage from the cells of *S. griseus*.

When sodium chloride was added to the cerelose-beef extract–peptone medium, it produced various effects on lysis of a shaken culture, depending on the salt concentration.

When no salt was added, there was no growth in 24 hours, and even the inoculum was lysed. With M/10 salt, there was some early growth, but it lysed by 24 hours. Infected cultures with M/5 salt (approximately 1 per cent) grew at the same rate as a control uninfected culture, and did not lyse in 5 days. Similar results were obtained by Walton[10] with a phage of *S. griseus*.

The fact that so many salts can increase the adsorption of phage, some even better than calcium, but that calcium cannot be replaced by other salts, indicates a role for calcium other than that of increasing phage-cell contact.

Discussion

Welsch's medium contains peptone, meat extract, dipotassium phosphate, and sodium chloride. I assume that the concentrations are about 0.1 per cent of phosphate and 0.5 per cent of sodium chloride, after similar media used by Waksman.[11]

If the effects of phosphate and other salts on the phages of actinomycin-producing streptomycetes are similar to their effects on phages of *S. griseus* and *E. coli*, we have the explanation of many of the difficulties in the isolation of lysogenic cultures listed above. The presence of the phosphate and sodium chloride would explain immediately: (1) poor adsorption of phage to cells; (2) recovery largely of sensitive isolates from secondary growth in lysed cultures; (3) carrying of the phage by some "partly resistant" colony isolates through several transfers; (4) failure of small phage inocula to lyse broth cultures of cells completely; (5) decrease in phage yield with increasing age of cell inoculum; (6) failure to account for as much as 50 to 80 per cent of the phage added in some experiments; and (7) irregularity in size and shape of plaques of some phage strains.

The emergence of partly resistant cultures (strains that produce reduced final crops of phage and that allow only a few plaques to develop on agar plates) cannot be explained with complete certainty on the basis of these experiments. However, at least two speculations may be advanced.

Some cultures may have altered slightly the phage-adsorption characteristics, so that the salts in the medium permit even lower rates of infection than

they do with the parent. Presumably, a medium could be found in which these strains would be completely susceptible.

Other cultures may deaminate the amino acids in the medium at a faster rate than the parent. The *p*H would rise sooner, precipitating the available calcium at a faster rate, making for lower phage crops and smaller plaque numbers.

Several alterations of the medium might be suggested. First, added phosphate, if it is necessary at all, should be kept to the minimum level required for a reasonable rate of growth. The peptone and meat extract may be replaced largely by a small amount of sugar or starch. A slowly metabolized carbohydrate would be preferred, to keep the *p*H of the agar above 5.75. As a buffer against a rise in *p*H, ammonium salts would serve in two ways. First, the utilization of some of the ammonium ion by the culture tends to lower the *p*H. Second, the remaining ammonium ion would buffer the medium against the basicity of free ammonia in the *p*H range 7 to 8. The concentration should not be so great as to cause desorption of the phage from cell surfaces.

Calcium salts might be added as the citrate, or possibly gluconate, to prevent their precipitation during an experiment, but should certainly be added in some effective form. All other salts should be avoided unless their presence is absolutely required.

Summary

The activities of a phage of *S. griseus* are affected by the composition of the medium in which they are mixed with the host cells. The primary controlling conditions are *p*H, availability of calcium, and the concentrations of other salts. The other ingredients are important insofar as they contribute to the raising or lowering of *p*H, or to the sequestering or precipitation of calcium.

The phage does not multiply in its usual latent period when the *p*H drops below a value between 5.5 and 5.75. In broth, the phage is active at a *p*H of 7.6. In agar, which contains phosphate, increasing the *p*H of the medium above 6.8 tends to decrease plaque size, probably by precipitating the calcium during incubation of the plates over long periods.

Calcium is required for the first few minutes of the infection cycle in some step other than adsorption. All the divalent cations promote adsorption of phage to cells at a concentration near M/1000, and the monovalent cations at a concentration near M/100. At higher salt concentrations the cations interfere with phage adsorption by the cells.

The usual media used for plating do not have sufficient calcium for maximal phage activity. Supplements of calcium increase the size and numbers of plaques obtained.

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Discussion

M. Welsch (*Université de Liège, Liège, Belgium*): In his contribution to the discussion of my paper, W. Gold pointed out that actinophage production and efficiency of plating are quantitatively influenced by the composition of the culture medium, in particular by *p*H and the nature and concentration of salts. He suggested that my observations probably were made under sub-optimal conditions and that this might explain: (1) the temporary lack of phage sensitivity of the indicators and, therefore, the occurrence of pseudolysogenicity, and (2) the failure to obtain artificial lysogenization.

While agreeing that optimal conditions for all the variables involved in phage production and enumeration were not sought, I reiterate that the point I wanted to stress was that pseudolysogenicity can and does occur under several sets of widely used experimental culture conditions, and should not be confused with true lysogenicity, as it was upon several occasions in the past.

Furthermore, I do not think that increasing the actinophage efficiency by a factor of 1.5, 2, or even 3 would alter my results significantly since, in many of my experiments, the number of *actually active* phages was in very large excess (100 or 1000 times) with respect to the number of indicator cells.

In his final comments, Gold stressed certain parallels between heterokaryosis and phage infection.

In fact, infection of a microorganism by the desoxyribonucleic acid (DNA) of a compatible bacterial virus often has been compared by several students of bacteriophagy to the fertilization of an egg by the spermatozoon, especially in view of the fact that such DNA, once introduced into the bacterium, from then on assumes a predominant role in directing the metabolic activities of the cell.

The analogy is even more striking in lysogenicity, when the viral DNA, instead of being multiplied in the cytoplasm of the infected cell, is reduced to the state of a prophage that in all probability is incorporated into the cell nucleus and transmitted to its progeny along with its own genome. As is well known, in such a case the lysogenizing virus occasionally carries with it and introduces into a few cells a given genetic determinant (transduction). Sometimes it even induces the appearance of a new heritable property in any and all cells in which it has established itself as a prophage (lysogenic conversion).

The presence in phages of a highly specialized structure ensuring penetration of the virus DNA into susceptible organisms has, however, no presently known counterpart in the mating phenomena of streptomycetes.

Elwood B. Shirling (*Department of Botany and Bacteriology, Ohio Wesleyan University, Delaware, Ohio*): It is appropriate that the phages of streptomycetes should be considered in this monograph dealing with the genetics of antibiotic-producing microorganisms. Although these phages were noted as early as 1936 by Wiebols and Wieringa,[1] and their apparent widespread occurrence in soils or even in the dust of the air has been verified by many investigators,[2-6] their significance to genetic, physiological, and taxonomic studies of actinomycetes has not been explored adequately. Paradoxically, this probably is due in part to the development of standard phage techniques.[7] Streptomycetes are not "standard" bacteria, and bacteriophage specialists have been inclined to work only with organisms that conform to the rules. Nevertheless, some of the more recent studies on *Streptomyces* phages, such as M. Welsch's paper on lysogenicity in *Streptomyces*, provide a reasonable basis for interest in possible genetic roles for the temperate actinophages. Incentives to pursue this line of thought are strengthened when it is remembered that the characteristic acquired by avirulent *Corynebacterium diphtheriae* upon lysogenization by the temperate β phage is ability to produce a metabolic product (toxin),[8-12] and that traits transduced in *Salmonella* involve fermentative capacities, nutritional requirements, drug resistance, and antigenic specificity.[13] The use of phage to transfer such genes among the antibiotic producers would be exciting, indeed; however, to my knowledge it has not been done. Conservatism forces us to recognize that transduction is an uncommon phenomenon among the many bacteriophage systems that have been studied. Perhaps the most encouraging thing that can be pointed out at this time is that the gross mechanism for gene transfer by phage appears to be present. The actinophages, as will be shown later are true analogues of the eubacterial phages, and some actinophages have the extremely wide host range desirable for transduction experiments.

Comparison of Actinophages and Eubacterial Phages

Morphologically, the phages of the streptomycetes are similar in considerable detail to such well-known phages as the T phages of *Escherichia coli* or those of the *Staphylococcus*. For example, the actinophages of Woodruff *et al.*[3] were described as having heads 50 mμ in diameter and tails bent and long, about 150 $\times$ 15 mμ. Abe *et al.*[14] described a larger phage with a cocoonlike head approximately 120 $\times$ 70 mμ and a tail 160 to 200 mμ in length and 30 mμ in width. Our phage[15] for *Streptomyces* S-77 had heads 68 to 70 mμ in diameter and curved or S-shaped tails 170 mμ long and 20 mμ in diameter. By comparison, the T 5 phage for *E. coli* has a head 65 mμ in diameter and a tail 170 $\times$ 10 mμ.

Empty coats analagous to bacteriophage coats described by Levinthal and Fisher[16] have been found.[17] Lysis of the host (within limitations imposed by a mycelial structure) follows the same pattern in actinomycetes as in eubacteria although, admittedly, experiments establishing the length of latent period and burst size are more difficult to perform. Electron micrographs have been published[15, 17] showing release of great numbers of phage particles both from terminal portions of hyphae and from internal sections of the mycelium of lysogenic S-77.

Plaques produced by actinophages on streptomycetes are remarkably like those of eubacteria, particularly when the host streptomycete seed layer is composed of newly germinated conidia. The familiar plaque types (clear, turbid, rapid-lysing, and minute) have all been observed in my laboratory, as doubtless they have in others. Plaque-type mutations are common, as are host-range mutations.[18] Thus, the gene markers for classic phage-recombination studies[19-21] are available in actinophages.

Values obtained by Welsch for thermal inactivation[22] and ultraviolet inactivation[23] of his streptomyces phage φ-17 are in agreement with representative values given for bacteriophages.[24]

Polyvalence of Certain Streptomyces Phages

In the first paper clearly to describe phage action on actinomycetes, one of the phages reported by Wiebols and Wieringa[1] was described as active against more than a single species; hence it may be assumed that it was polyvalent. Phages reported for *Streptomyces griseus* fermentations in 1947, a decade later, for the most part were found to be specific for streptomycin-producing strains.[2-5] In fact, one of these specific phages was used for rapid identification of streptomycin-producing strains of *S. griseus*.[25] In 1953, Carvajal[26] tested the host range of his *S. griseus* phage against species representing 5 genera of the order Actinomycetales, including 84 cultures of *S. griseus*. Of the latter, 66 were streptomycin producers and 18 nonproducers. All but five of the streptomycin producers were sensitive. Of the 18 nonstreptomycin producers, 2 griseinproducing cultures were sensitive, while the others were insensitive. Twenty-one additional species of *Streptomyces* from the collection of the Northern Regional Research Laboratory, Peoria, Illinois, were resistant to the phage, as were *Nocardia corallina* ATCC-999, *N. asteroides* JS-10, *N. farcinica* ATCC-3318, *Actinomyces bovis*, *Micromonospora* sp. ATCC-10026, *Mycobacterium tuberculosis*, *M. phlei*, and *M. smegmatis*.

More remarkable, however, was the fact that 3 species in addition to *S. griseus* were susceptible to the phage. They were *S. olivaceus* NRRL B-1125, *S. griseolus* ATCC-3325, and *S. viridis* ATCC-3372.

Welsch *et al.*[27] indicate that the phages they have isolated display a relatively low degree of specificity. In a later report Welsch *et al.*[28] showed that their phages φ-17 and φ-17/5 are active against 8 strains of *S. griseus* Krainsky and against practically all organisms in their Group IV of actinomycin producers (but not against Groups I, II, III). *S. chrysomallus* Lindenbein and *S. parvus* NRRL B-1455 are included in their Group IV. This suggestion of polyvalence is not discounted by the authors' decision that all of the organisms susceptible to these phages should be considered as one species, *S. griseus*. There was a considerable range of variation in a number of properties, including antibiotic production, among the cultures sensitive to the phage.

During the past 18 months we have isolated 14 phages on as many different *Streptomyces* hosts. Each phage was purified by 3 or more single-plaque isolations. In order to determine host-range over a very broad spectrum, these phages were then tested on representatives of 5 of Pridham's sporophore morphology sections of the genus *Streptomyces*.[29] Test cultures were supplied

from the NRRL collection by Pridham. Host ranges for 4 of these phages are shown in TABLE 1.

In addition, phage OP-8 was active against 8 of 10 unnamed test cultures of *Streptomyces*, and phage OP-14 was active against 6 of these cultures. Thus, regardless of the validity of sporophore morphology in speciation, it is evident

TABLE 1

HOST RANGES OF STREPTOMYCES PHAGES WITH RESPECT TO REPRESENTATIVES OF DIFFERENT MORPHOLOGIC SECTIONS OF THE GENUS *STREPTOMYCES*

Hosts: section and species	Phages			
	OP8	OP12	OP14	OP1
Section Rectus-flexibilis				
S. griseus NRRL B-150	−	+	+	−
S. venezuelae NRRL B-902	−	−	−	−
Section Retinaculum-apertum				
S. lavendulae NRRL B-1230	−	−	+	−
S. fradiae NRRL B-1195	+	−	−	−
Section Spira				
S. viridochromogenes NRRL B-1511	+	−	+	−
S. hygroscopicus NRRL B-1346	+	−	+	+
Section Biverticillus				
S. cinnamomeus f. cinnamomeus NRRL B-1285	+	−	+	−
S. rubrireticuli NRRL B-1707	+	−	+	−
Section Biverticillus-spira				
S. netropsis 2268	+	−	−	−

Symbol + indicates lysis by a spot test using phage titer between 1×10^8 and 1×10^9 particles per ml. assayed on original host. All phage reactions in the table were verified by plating out suitable dilutions to give true plaques on each host giving a positive spot test.

TABLE 2

ASSAY TO DETERMINE EFFICIENCY OF PLATING OF POLYVALENT PHAGES ON VARIOUS HOSTS

Phage stock and titer on propagating host*	Assay on various hosts*				
	S. griseus NRRL B-150	*S. viridochromogenes* NRRL B-1511	*S. hygroscopicus* NRRL B-1346	*S. cinnamomeus f. cinnamomeus* NRRL B-128	*S. rubrireticuli* NRRL B-1707
OP8 (3.2×10^8)	0	9×10^6	3.2×10^7	3.7×10^6	1.3×10^8
OP14 (2.4×10^7)	1×10^5	1.2×10^4	8×10^6	1.9×10^6	1.8×10^6

* Plaque-producing particles per ml.

that some phages are active against a very broad spectrum of streptomycete hosts, whereas others are limited in host range. However, the efficiency of plating for polyvalent phages varied on different hosts, as shown in TABLE 2.

The observation that some phages have a limited host range, whereas the infectivity of others may even cross generic boundaries, is not new. For example, Adams and Wade[30] show that the serologically related phages T3, T7, and I, all infect strains of *E. coli*, *Shigella sonnei*, and *Serratia marcescens*. Sim-

ilarly, Bradley and Anderson[31] have isolated 3 phages on *Streptomyces* that are not only active on several members of this genus, but also attack members of the genus *Nocardia*. Conversely, 3 phages isolated on *Nocardia* were active against several streptomycetes. This may or may not pose questions about the validity of present taxons, but it does furnish additional documentation of polyvalence in some actinophages.

Polyvalence among strictly virulent phages presumably will not furnish a mechanism for transductive gene transfer. It is significant, therefore, that we are finding that some of our streptomycete phages with extended host range also show a range in lytic activity against the various hosts. Duplication of conditions found in *Salmonella* transduction will require a streptomycete carrying a lysogenic phage with weakly lytic activity against the proposed donor streptomycete. While these conditions have as yet not been fulfilled experimentally either through artificial lysogenization of streptomycetes or through use of naturally lysogenic systems, the possibility that some anomalies in genetic behavior of actinomycetes may be due to phage-carried DNA cannot be ruled out. The close similarity between actinophage and better-known bacterial phages should encourage a search for true transduction phenomena in the actinomycetes.

Phages and Variation in Streptomyces

Three independent studies emphasize phenotype variation expressed by certain streptomycetes exposed to, or carrying, actinophage. Carvajal, reporting in 1953[26] and Welsch, in 1957,[32] described atypical colonies developing as survivors after exposure to virulent phage; Shirling[17] found a wide range of variants, remarkably similar to those described by the above-mentioned authors, among the progeny of a naturally lysogenic streptomycete (FIGURE 1). In the latter case, when dilute spore suspensions of the normal-appearing, but lysogenic streptomycete were plated by the soft agar–layer technique to give isolated colonies, 20 per cent of more than 5000 colonies were distinct variants (FIGURE 1*a*). In individual experiments, each involving several hundred colonies, variation ranged from 5 per cent to 33 per cent of the total. Certain soft types of variants (FIGURES 1*a*, *f* and 2*a*) were found to produce more phage lysogenically than the normal colonies. Normal-colony types continued to give rise to variants through many transfers and single-colony isolations over a period of 6 years. Soft-colony types have never reverted to normal types (FIGURES 2*d* and 3). Atypical colonies were in higher proportion when the dilution was great enough to produce only a few colonies per plate. In separate experiments the ratio of atypical to typical colonies increased greatly when cultures were grown first in broth on a shaker and survivors were plated, or when cell-free filtrate containing phage from the extremely soft and irregular variant was added (FIGURE 2*c*). In this respect the lysogenic culture behaved much as do survivors of sensitive cultures grown in broth in the presence of virulent phage.

In the case of virulent phage, when an excess of phage is plated with a suitable inoculum of a sensitive streptomycete host, a surviving population often gives rise to a number of "resistant" colonies, which may or may not be strikingly

different in appearance from the host. We have noted this abundance of survivors on plates prepared with 12 different phage isolates on a wide range of hosts. Similar results can also be obtained by plating from broth cultures grown with phage on a shaker for 48 hours. Rarely do such cultures become

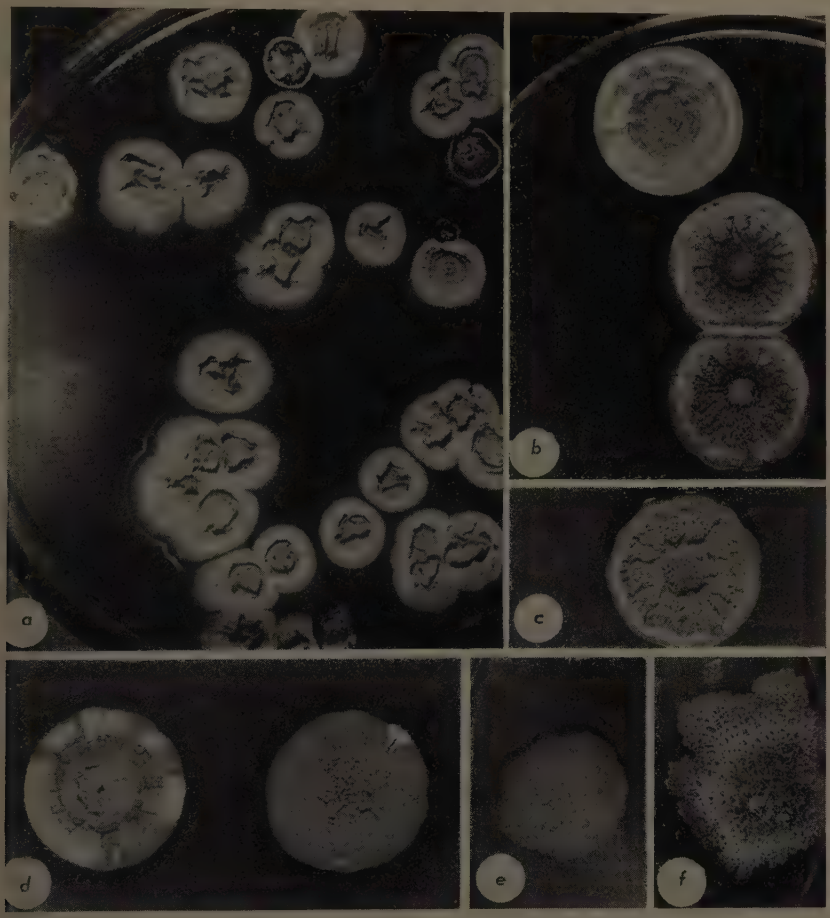

FIGURE 1. Variation in colonies plated from agar-slant culture of *Streptomyces* S-77. Colonies had developed two weeks on glycerol-nutrient-agar: *a*, soft and asporogenous colonies among normal, coriaceous, sporogenous types; *b* through *f*, colonies selected to show range of variation obtained when colonies were widely spaced (less than 6/plate). Rugose or lacy folded areas are soft and partially lysed.

cleared of growth by phage action. In many instances the shaken broth culture is turbid, resembling eubacterial growth; in other cases the mycelial fragments that resist lysis form a pelleted growth in the broth. This resistant growth often will give rise to a variety of colony types when plated on agar medium. Such variants have been described by Carvajal and Welsch for streptomycin-producing *S. griseus* and an actinomycin-producing streptomy-

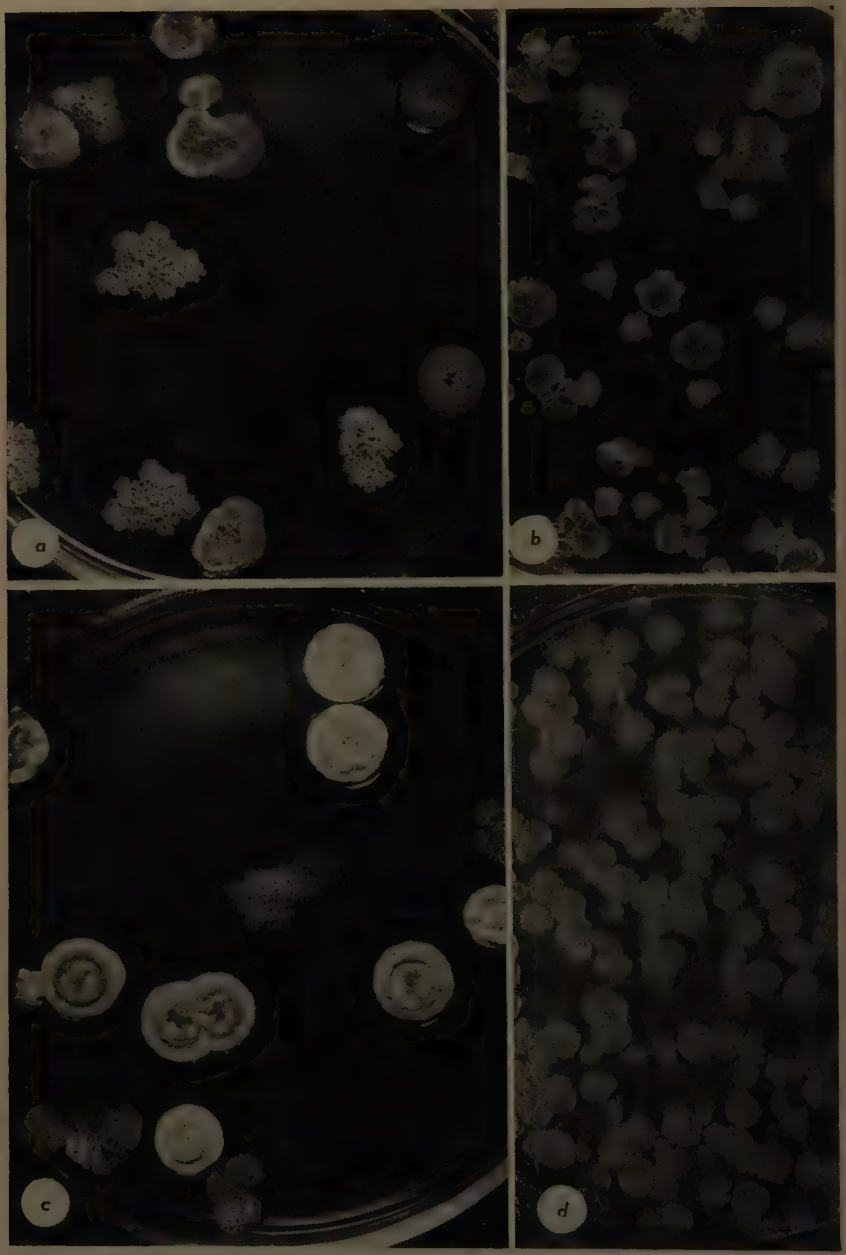

FIGURE 2. Two soft-colony types derived from the soft colony at left side of Petri plate in FIGURE 1*a*. Variants in FIGURE 1*a* were isolated on agar-slant stock cultures and carried through several transfers before plating. FIGURE 2, *b* and *d*, result of isolating two colony types in *a*; *c*, result of plating agitated broth culture of normal S-77 grown in the presence of cell-free phage filtrate from broth culture of soft variant. Addition of phage increases ratio of soft to normal colonies.

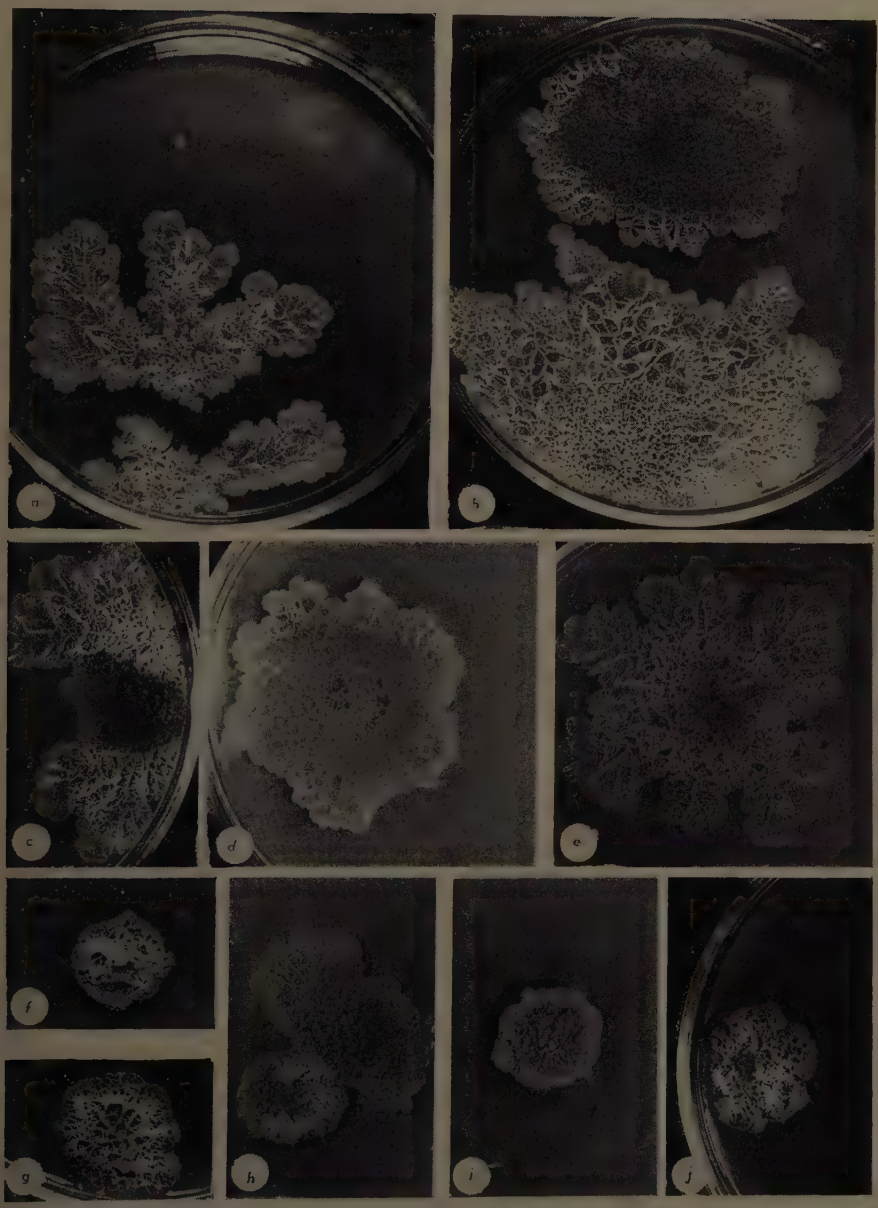

FIGURE 3. Range of variation obtained from a single stock culture of the soft variant shown in FIGURE 2*c*. Colonies had developed 3 weeks on glycerol-nutrient agar. Greatest variation is seen when inoculum is diluted to give less than 6 colonies/plate (compare with FIGURE 2*b*). Reversion to normal-colony type has never occurred with the soft variants, but FIGURE 3*f*, *g*, *i*, and *j* represents colonies with a friable to hard consistency, adherent to the agar, and less spreading than the typical soft type.

cete (S-17), respectively. Carvajal's *S. griseus* phage also resulted in unusual colony types among survivors of *S. olivaceus* NRRL B-1125.

Variants described by the three authors cited above included minute colonies that usually did not survive; soft, yeasty, or lacy asporogenous growth; crumbly, asporogenous or mottled growth; and normal-appearing colonies. They also differed from one another in physiological properties such as antibiotic production and pigmentation. The changes from the parent strain were sufficiently striking to cause Carvajal[26] to regard the phage as a mutagenic agent comparable to X rays, ultraviolet light, and nitrogen mustard gas. More recently, Alikhanian and Iljina[33] have made similar observations and have come to a similar conclusion regarding action of a phage on *Actinomyces olivaceus* N-6. Although they regarded the phage as a mutagenic agent, they also noted an influence of the propagating host on the "mutational spectrum" of the phage. When the phage was grown on *S. griseus* Strain II, white, light yellow, and lemon yellow variants were induced in *S. olivaceus* exposed to the phage action. However, the same phage propagated on *S. olivaceus* induced black and brown colonies. The suggestion made by these investigators that this phenomenon is comparable to transduction in bacteria is not entirely compatible with the idea that the phage is a simple mutagenic agent.

Welsch has provided good evidence that, with his actinomycin-producing S-17, the changes are not accompanied by an acquired lysogenicity, since surviving cultures of this streptomycete can be freed of phage and are often sensitive to the phage. On the other hand, through mass transfer of the secondary growth that survived phage action, actinophage and streptomycete were propagated together through many subcultures in a more or less stable balance. Welsch noted the emergence of semiresistant types that were variable in their sensitivity to the original phage; he noted also that in these types the propagated phage differed from the infecting phage either in ability to produce plaques on the homologous strain or in host range.

It is obvious that more study is needed before we shall be able to untangle the genetics of actinomycete host and actinophage. Correct interpretation of the factors that result in the wide variations to be found in phage-carrying streptomycetes or in cultures exposed to phage action offers an interesting challenge.

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Elizabeth McCoy (*University of Wisconsin, Madison, Wis.*): It is always stimulating to read a paper on a subject of one's own recent interest, and it is satisfying to discover many points of agreement between the author's work and ones own, although descriptions of both are as yet unpublished. This is the situation with respect to M. Welsch's paper and my own work and thoughts on the subject.

I am very glad to see that Welsch is conservative in his definition of lysogenicity, limiting it to the carrying of the phage in the prophage state, rather than describing it as free phage in low titer in a so-called carrier state dependent upon balanced propagation of phage in a semiresistant population. Robert Otto in my laboratory undertook to lysogenize *Streptomyces griseus* with our phage W2a, but he succeeded only in establishing the carrier-type lysogeny. To the casual observer this was lysogeny, the phage being detectable for 20 transfers but always in low titer, such as 10^2 and 10^3 particles/ml., as detected by plating filtrates upon a sensitive indicator strain; the titer was determined upon the parent host strain, *S. griseus* 1947, which itself is sensitive and able to propagate phage W2a to a titer of 10^8 or higher.

I am interested in Welsch's technique for confirmation of true lysogeny. My associates and I likewise were forced to prove whether true lysogeny was involved in our cultures before coming to the conclusion that we were dealing with the carrier-type lysogeny. Our tests were threefold:

(1) Analysis of the population by a replica-plating technique upon agar with and without added W2a phage. Result: within 10,000 colonies tested, 2 were found typically sensitive and all others were fully and typically resistant (that is, free of phage and unable to propagate it upon addition of more W2a phage). The 2 sensitive strains did propagate the phage to a titer of 10^8 or higher.

(2) Interruption of the phage cycle of reproduction as a test of extracellular phage. Our W2a phage with *S. griseus* requires Ca^{++} for adsorption. By sequestering Ca^{++} with Na oxalate, 0.008 *M*, the free phage in our carrier lysogenic cultures was removed in 1 to 2 transfers, whereas in the presence of Ca^{++} (0.005 *M*) it was carried for at least 20 transfers. In the Ca^{++}-free cultures in later transfers, for example the fifth, Ca^{++} was again added with no evidence of phage in the culture then or later. We considered this observation to imply that there was no prophage involved. Cultivation for a few transfers in the absence of Ca^{++} would seem, with *S. griseus* at least, to be a means of eliminating low-grade phage—purifying a culture, if one may call it so—before inducing a latent prophage and achieving a single entity of that phage in the filtrate. Welsch's suggestion that an antiphage serum could be used also occurred to us, and we used that approach also to distinguish the carrier from the true lysogenic state. I think that, of the two techniques, the control of the Ca^{++} and thus of adsorption is the more precise.

(3) Attempts to induce the prophage, if present, with mutagenic agents and thus to raise the titer of free phage were made. Our experiments in this line were not extensive; they were limited to ultraviolet irradiation and were not successful. Thus, we have only negative evidence to offer with the system *S. griseus* phage W2a, but we did have experience with another strain of *S. griseus*, C 131. This strain was lysogenic for a phage that was liberated with our W 1 phage, when W 1 was propagated upon Strain C 131. For a time this was very confusing in the characterization of C 131 by host pattern and serologic analysis, but it was later explained by inducing liberation of the C 131 phage in virulent state in sufficient titer to plate upon other strains of *S. griseus*, with typical plaques resulting. This is our only experience, thus far, with a naturally occurring lysogenized host. It seems to answer all of the criteria for a prophage-defined lysogeny.

It might be worthwhile to mention that our experience agrees with that of Welsch: we know of no sure method for induction of prophage in the streptomycetes. We generally use a mixed culture of the suspect strain and a number of possible indicator strains, relying either upon serial transfer or the presence of mutagenic treatments to stimulate release of the carried prophage and its propagation to easily detectable titers. This is a cumbersome method and is convincing only when results are positive.

In conclusion, I shall comment upon one problem in the study of streptomycete lysogeny or, for that matter, of any other problem of phage attack upon streptomycetes. The mycelial nature of the vegetative body presents problems of heterogeneity of the phage-sensitive host. The branched mycelium actually represents a gradation of ages of tissue, and we have seen in phase microscopy and also in electron microscopy that the tips of branches may be lysed off, leaving fragments of still viable primary mycelium. These are apparently still sensitive, since they are so upon transfer to new medium, with phage added immediately or after 4 to 8 hours, when they are in an active growing state. This survival of the aged mycelium, even in the presence of massive doses of phage, makes it very difficult to derive resistant strains by plating survivors. Because this aged mycelium still can adsorb phage (but not propagate it), there is danger of misinterpretation of the presence of phage in subcultures. In making serial transfers in broth, it might be thought even that the carried phage was in lysogenic relation, if the transfers were large in volume and not carried beyond dilution to extinction.

One is tempted to think, too, of the genetic complications that might arise in phage work done with vegetative mycelium of the streptomycetes. What complications in interpretations of sensitivity, resistance, and lysogeny, as well as stability in any one state, would one encounter with phage-host relations in a heterokaryotic host! For this reason we never use even so-called young vegetative material in our phage work. We use spores in an early stage of germination in an especially designed medium with optimum Ca^{++}, incubated 4 to 6 hours on a rotary shaker. Under our conditions we can achieve almost maximum germination, and the germ tubes are quite uniform, usually about 2 to 3 times the length of the spores and still unbranched. Such material is highly sensitive, reproduces the phage to high titer, and is very reproducible from experiment to experiment. I am pleased to note from other papers in this monograph that the nuclear apparatus of such germinating spores should be genetically good material for manipulation. Even if the spores in the suspension are heterogeneous, selection by plating for either lysogenic or resistant strains should be possible. Phage acting upon the host in the germinated spore state can be counted upon to destroy all, or nearly all, susceptible strains. Upon plating, we have recovered as high as 94 per cent of true resistant strains; the remainder are sensitive strains, perhaps ungerminated spores at the time of phage action and reverse mutants to sensitive state. In any case, it is very convenient that use of conidia allows one to assume a genetic uniformity of the host for phage studies. Conversely, application of phage to the germinated spore stage of the host may offer, genetically speaking, a technique for analysis of the nuclear potentials of a culture of streptomycetes.

S. G. Bradley* (*Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minn.*): The streptomycetes are well known, principally because they produce substances that kill or prevent the growth of pathogenic microbes. Most of these antimicrobial substances are relatively simple organic compounds that inhibit preferentially molds, protozoa, or bacteria. In addition, many streptomycetes liberate agents that specifically destroy members of the genera *Nocardia* and *Streptomyces*.[1] These agents, designated actinophages, morphologically resemble bacteriophages.[2] Organisms that possess the hereditary capability to produce actinophages are referred to as lysogenic.

A strain reasonably may be considered lysogenic if the power to liberate actinophage is retained after several clonal subcultures.[3] Lysogeny is not established definitively unless the capability to yield actinophage persists after growth of the streptomycete in a medium preventing infection by free virus.[4] Exogenous infection can be prevented by adding to the nutritive substrate an antiserum specific for the particular temperate actinophage. These rigorous criteria must be met satisfactorily in order to distinguish lysogenic strains from carrier strains.[5] Carrier strains are mixed populations in which the majority of the bacteria are resistant; the infrequent sensitive variants, however, become infected with, and support growth of the exogenous actinophage. In the streptomycetes, differentiation between truly lysogenic strains and carrier populations is complicated because conidiospores, unsporulated aerial hyphae, and vegetative hyphae often display diverse susceptibilities to a particular actinophage.[6] The vegetative mycelium, moreover, is heterogeneous in this regard, probably because the growing hyphal tips and the older intercalary segments differ significantly in physiological age.[7]

Induction

Evidence for lysogeny is obtained by plating bacteriologically sterile culture filtrates with suitable indicator strains.[8] The probability of detecting spontaneously induced temperate actinophages frequently is not increased by growing the strain to be tested with a sensitive indicator strain; the density of free actinophage in the culture filtrate, however, may be increased substantially by this procedure.[9] I have tried unsuccessfully to induce the liberation of virus by treating lysogenic streptomycetes with penicillin G, azaserine, ultraviolet irradiation, and hydrogen peroxide, alone and in various combinations;[10] similar attempts by other investigators to obtain a reproducible method for induction also have failed (Welsch, elsewhere in this monograph). Regular induction of a temperate actinophage has been obtained only as a result of superinfection with a virulent virus.[11]

Distribution of Lysogeny

Inasmuch as induction is irregularly successful, the distribution of lysogeny in nature can be studied only with difficulty. Even so, I was able to demonstrate free actinophage in culture filtrates from 9 of 17 serially recloned strains

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of streptomycetes.[10] The hereditary capability to produce actinophage of 3 of these 9 strains was unaffected by growth in medium containing antiserum specific for the appropriate virus. Shirling has studied the cohabitation of actinophages with streptomycetes; on the basis of studies using the electron microscope, he concluded that about 40 per cent of fresh isolates of *Streptomyces* carry virus. Unfortunately, he was unable to obtain sensitive indicator strains for any of these viruses.[12] Clearly, lysogeny is widespread among the streptomycetes; undoubtedly, many strains not now considered lysogenic will be found to contain temperate actinophage as soon as suitable methods for induction are known.

Lysogenization

Experimental lysogenization has rarely been achieved with the streptomycetes.[11] Although many colonies developing after the lytic action of actinophage continue to liberate virus during a limited number of subcultures, the survivors are carriers rather than truly lysogenic. Carrier systems resulting from attempts to lysogenize artificially are especially stable because resistant and partially resistant mutants are selected by the added actinophage.

The capability of a temperate phage to lysogenize a sensitive host is controlled by three factors: the genetic composition of the phage, the genetic composition of the host, and the environment.[5] Because many attempts to lysogenize have been conducted with known temperate actinophages, the genetic composition of the virus probably is not the factor responsible for present failures. Certainly, the physiological heterogeneity within a streptomycete population is a deterrent to controlled studies. Moreover, many streptomycetes produce substances toxic to actinophages;[13] the survivors of actinophage, therefore, may be able to free themselves partially or completely of the virus. Unfortunately, the streptomycetes known to produce antiphage substances have not been tested for lysogenicity. If a lysogenic strain synthesizing antiviral chemicals is found, the suceptibility of the respective temperate actinophage to these antibiotics will be of considerable interest.

The reverse of lysogenization probably has been observed on one occasion in my laboratory. A lysogenic streptomycete, resistant to its temperate actinophage, yielded spontaneously a variant sensitive to the virus. Free actinophage has not been recovered from the culture filtrates of this presumably delysogenized organism.

Immunity

Lysogenic eubacteria are resistant to the respective temperate phages.[5] These viruses can attach to and penetrate the cell wall of the lysogenic strain, but the parasite is unable to initiate a progressive infection. Similarly, some lysogenic streptomycetes are immune at all growth stages to their temperate phage. Conversely, other lysogenic streptomycetes seem to be sensitive to the respective temperate actinophage.[10] Newly germinated spores are frequently destroyed by the temperate phage for which they are lysogenic. An alternative explanation to account for the formation of plaques by temperate phage on the respective lysogenic streptomycete is that the added virus acts as an inducer.

Lysogenization usually confers two new characteristics on the recipient: the hereditary capability to produce phage, and resistance to exogenous infection

by that phage. The dual function of the virus-gene complex is not mandatory; for example, resistance without capability to liberate temperate phage has been described in *Escherichia coli*. The presence of a noninducible temperate bacteriophage was demonstrated as a result of recombination with an inducible temperate phage.[5] The converse, that is, the hereditary power to produce phage without concurrent resistance, is conceivable.

Application of Lysogeny

The ability of streptomycetes to produce antibiotics is correlated with susceptibility to actinophages.[8] Such a relationship calls to mind the role of temperate bacteriophage in toxigenic conversions with *Corynebacterium diphtheriae*.[14] Moreover, the capability of certain temperate phages to serve as vectors in the transfer of a limited amount of genetic information from a disrupted donor to an intact recipient cell is well recognized.[15] Application of these methods conceivably can provide the fermentation industry with strains (1) yielding practical levels of two or more useful antibiotics simultaneously, (2) producing new or altered antibiotics, or (3) able to perform new chemical transformations. Finally, a study of lysogeny in streptomycetes may reveal unfamiliar aspects of virus-host interactions; for example, it is well established for many lysogenic systems that the free phages arise as a result of bacterial lysis of only a small proportion of the population, but the concept that phages are secreted continuously during growth should not be considered impossible.[16]

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